2000

Isolation and Characterization of Newly Emerging Coronaviruses in Acute Respiratory Tract Diseases of Cattle.

Xiaoqing Lin
Louisiana State University and Agricultural & Mechanical College

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

ISOLATION AND CHARACTERIZATION OF NEWLY EMERGING CORONAVIRUSES IN ACUTE RESPIRATORY TRACT DISEASES OF CATTLE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through
the Department of Veterinary Microbiology and Parasitology

by
Xiaoqing Lin
M.D., Shanghai Medical University, Shanghai, P.R. China, 1990
May, 2000

DEDICATION

To my parents, Mr. Songbo Lin and Mrs. Fudi Wang. Your patience, love, sacrifice and support have allowed my dreams to come true.

To my uncle, Dr. Ruiliang Lu. Your continued support and encouragement have made me to achieve my goals.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my major professor, Dr. Johannes Storz, for giving me the opportunity to further my education in Biomedical Sciences, to develop my talents and to reach my ultimate goal of becoming a physician-scientist. I am greatly indebted to Dr. Konstantin G. Kousoulas for his advice on molecular virological aspect and the use of the Gene Lab facilities, and to Dr. Kathy L. O'Reilly for her immunological guidance. My thanks are also extended to the members of my graduate committee, Dr. Sue G. Bartlett, Dr. Elmer K. Godney and Dr. Robert Strongin, for their critical advice.

I greatly appreciate the help of Dr. Robert E. Truax for supplying excellent special cell culture, which was an invaluable contribution to these investigations. Special thanks are due to Drs Vladmir N. Chouljenko, Richard E. Corstvet, Abolgasem Baghian and Timothy P. Foster for their expert help and support in various experiments, which contributed tremendously in unraveling this "emerging" respiratory bovine coronavirus story.

I thank Dr. William G. Henk, Laura Younger and Gregory McCormick for their help with experiments using phase-contrast, electron or fluorescence microscopy, and digital analysis. I am grateful to Micheal Kearney for statistical evaluation. The

technical assistance of Mamie Burrell, Michael L. Broussard, Lindy Belle Cox, Jenny Marie Arcement, Li-jun Huang and Patricia Triche is gratefully acknowledged.

I especially appreciate the contributions of my family, Mr. Songbo Lin, Mrs. Fudi Wang, Dr. Ruiliang Lu, Mr. Xiaomin Lin and Mrs. Liqin Fang, who provided me with intellectual stimulation and constant encouragement throughout my education.

These investigations were funded by grants from the Critical Issues and the National Research Initiative Programs of the United States Department of Agriculture (98-34362-6071, 94-37204-0926), the Louisiana Education Quality Support Fund (RF/1995-1998 RD-B-18) with matches from Immtech Biologics, LLC, Bucyrus, KS, and Bayer Corporation, Merriam, KS, the Texas and Louisiana Agricultural Experiment Stations (Regional Research NC107), the Texas Advanced Technology Program (Grant No 999902), the Louisiana Beef Industry Council, and the School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA.

TABLE OF CONTENTS

DEDICATION----ii

ACKNOWLEDGMENTS----iii

LIST OF TABLES----vii

LIST OF FIGURES----x

ABSTRACT----xii

CHAPTER

1 INTRODUCTION----1

1.1 Statement of Problem and Hypothesis----1

1.2 Research Objectives----2

2 LITERATURE REVIEW----5

2.1 Pathogenic Potential of Coronaviruses----5

2.2 Coronavirus Infections of Cattle----5

2.3 Molecular Properties of Bovine Coronavirus----9

3 DYNAMICS OF EMERGENCE OF RESPIRATORY BOVINE CORONAVIRUS INFECTIONS-A CATALOGUE OF VIRUS ISOLATES FROM MAJOR EPIZOOTICS----27

3.1 Introduction----27

3.2 Materials and Methods----31

3.3 Results----43

3.4 Discussion----93

4 ANTIBODY RESPONSES TO RESPIRATORY CORONAVIRUS INFECTIONS OF CATTLE DURING SHIPPING FEVER PATHOGENESIS----102

4.1 Introduction----102

4.2 Materials and Methods----106

4.3 Results----112

4.4 Discussion----123

5 INFECTION OF POLARIZED EPITHELIAL CELLS WITH RESPIRATORY AND ENTERIC TRACT BOVINE CORONAVIRUSES AND RELEASE OF VIRUS PROGENY----------130
5.1 Introduction----------130
5.2 Materials and Methods----------132
5.3 Results----------136
5.4 Discussion----------142

6 COMPARISON OF FUSOGENIC PROPERTIES OF RESPIRATORY AND ENTERIC TRACT BOVINE CORONAVIRUSES----------148
6.1 Introduction----------148
6.2 Materials and Methods----------150
6.3 Results----------154
6.4 Discussion----------159

7 HEMAGGLUTININ-ESTERASE SPECIFIED BY RESPIRATORY BOVINE CORONAVIRUSES HAS TEMPERATURE-SENSITIVE ACETYLESTERASE ACTIVITY----------165
7.1 Introduction----------165
7.2 Materials and Methods----------169
7.3 Results----------173
7.4 Discussion----------184

8 CONCLUSION AND PERSPECTIVES----------189

REFERENCES----------195

APPENDIX: LETTER OF PERMISSION----------214

VITA----------215

LIST OF TABLES

Table 3.1 Respiratory bovine virus isolation from nasal swab samples of cattle in Kansas Feedlot and Arizona Feedlot----------45

Table 3.2 Respiratory bovine virus isolation from nasal swab samples of cattle in the 1994 Louisiana State University livestock show---------52

Table 3.3 Respiratory bovine virus isolation from nasal swab samples of cattle in clinical cases----------53

Table 3.4 Respiratory bovine virus isolation from nasal swab samples of cattle of the Lawton Ranch herd----------54

Table 3.5 Respiratory bovine virus isolation from nasal swab samples of cattle at the order-buyer barn during the 1997 shipping fever epizootic----------55

Table 3.6 Respiratory bovine virus isolation from nasal swab samples of cattle on day 7 post-arrival at the order-buyer barn during the 1997 shipping fever epizootic----------60

Table 3.7 Respiratory bovine virus isolation from nasal swab samples of cattle on day 14 post-arrival at the order-buyer barn during the 1997 shipping fever epizootic----------63

Table 3.8 Respiratory bovine virus isolation from nasal swab samples of cattle on day 21 post-arrival at the order-buyer barn during the 1997 shipping fever epizootic----------66

Table 3.9 Respiratory bovine virus isolation from nasal swab samples of cattle at the order-buyer barn during the 1998 shipping fever epizootic----------69

Table 3.10 Respiratory bovine virus isolation from nasal swab samples of cattle on day 7 post-arrival at the order-buyer barn during the 1998 shipping fever epizootic----------73

Table 3.11 Respiratory bovine virus isolation from nasal swab samples of cattle on day 14 post-arrival at the order-buyer barn during the 1998 shipping fever epizootic----------78

Table 3.12 Respiratory bovine virus isolation from nasal swab samples of cattle on day 21 post-arrival at the order-buyer barn during the 1998 shipping fever epizootic----------82

Table 3.13 Isolation of respiratory bovine viruses and *Pasteurella spp.* from nasal and lung samples of cattle that died in the 1997 shipping fever epizootic----------86

Table 3.14 Loads of respiratory bovine coronaviruses and *Pasteurella spp.* in lungs of cattle that died in the 1997 shipping fever epizootic---------88

Table 3.15 Isolation of respiratory bovine viruses and *Pasteurella spp.* from nasal and lung samples of cattle that died in the 1998 shipping fever epizootic----------89

Table 3.16 Loads of respiratory bovine coronaviruses and *Pasteurella spp.* in lungs of cattle that died in the 1998 shipping fever epizootic---------90

Table 3.17 Isolation of respiratory bovine viruses and *Pasteurella spp.* from trachea and lung samples of cattle that died in the winter pasture-------92

Table 3.18 Correlation between respiratory bovine coronavirus infection and signs of respiratory tract diseases of cattle in the 1997 shipping fever epizootic----------94

Table 3.19 Correlation between respiratory bovine coronavirus infection and signs of respiratory tract diseases of cattle in the 1998 shipping fever epizootic----------95

Table 3.20 Frequency of virus isolations from nasal secretions of cattle with acute respiratory tract diseases----------96

Table 3.21 Frequency of virus isolations from lungs of cattle with fatal pneumonia----------97

Table 4.1 Respiratory coronavirus isolations and clinical signs of cattle in response groups of the 1997 shipping fever epizootic----------108

Table 5.1 Cytopathic changes in G clone cells following apical and basolateral exposure to respiratory and enteropathogenic bovine coronaviruses and vesicular stomatitis virus----------138

Table 5.2 Hemagglutinin titers released by G clone cells following directional exposure to 4 strains of respiratory and entero-pathogenic bovine coronaviruses----------140

Table 5.3 Titers of hemagglutinin and viral infectivities of virus after directional inoculation of G clone cells with respiratory and enteropathogenic bovine coronaviruses and vesicular stomatitis virus----------141

Table 5.4 Hemagglutinin titers and virus infectivities in harvest of G clone cells----------143

Table 7.1 Hemagglutinin and receptor-destroying enzyme activity of respiratory and enteropathogenic bovine coronaviruses----------174

LIST OF FIGURES

Figure 2.1 Model of coronavirus structure----------11

Figure 2.2 Organization of enteropathogenic bovine coronavirus genome----------13

Figure 2.3 Model of coronavirus replication----------19

Figure 2.4 Models for transcription of coronavirus subgenomic mRNAs----------23

Figure 3.1 Negatively stained viral particles of RBCV OK-0514-2----------50

Figure 4.1 Levels of total and isotype antibodies to respiratory bovine coronavirus in sera from cattle of response groups 1, 2, 3, 4 and 5 during the 1997 shipping fever epizootic----------114

Figure 4.2 Representative western blot analysis of sera from calf 97TXSF-105 of response group 1----------118

Figure 4.3 Representative western blot analysis of sera from calf 97TXSF-3 of response group 2----------120

Figure 4.4 Representative western blot analysis of sera from calf 97TXSF-88 of response group 4----------121

Figure 4.5 Representative western blot analysis of sera from calf 97TXSF-4 of response group 5----------122

Figure 5.1 Scanning electron micrograph of a monolayer of uninfected G clone cells----------137

Figure 6.1 Cytopathic changes in G clone cell cultures infected with EBCV-L9-80 (A) or RBCV-OK-0514-3 at 24 h postinfection (B)-----155

Figure 6.2 Assessment of membrane integrity of the RBCV-induced polykaryons by trypan blue exclusion----------156

Figure 6.3 Structural features and morpholoy of nuclei in RBCV-induce polykaryons after Giemsa staining----------158

Figure 6.4 Ultrastructural evidence for RBCV morphogenesis within RBCV-induced polykaryons at 12 h postinfection----------160

Figure 7.1 Cloning and expression of HE gene in COS-7 cells----------176

Figure 7.2 Serine-esterase activities associated with purified RBCV and EBCV, and expressed HE proteins from RBCV and EBCV----------178

Figure 7.3 Acetate release from bovine submaxillary mucin (BSM) by purified RBCV and EBCV, and expressed HE proteins from RBCV and EBCV----------181

Figure 7.4 Comparison of the predicted amino acid sequences of the HE proteins specified by different bovine coronavirus strains----------183

ABSTRACT

A total of 411 G clone cell-dependent virus strains recently were isolated from nasal, trachea and lung samples in etiological investigations of acute respiratory tract diseases of vaccinated cattle, including shipping fever pneumonia. The virus infectivity titers reached up to 1.2×10^7 plaque forming units per gram of lung tissues of fatal cases. These viral agents were isolated in the 1st G clone cell passage without trypsin enhancement, and induced cell fusion. They have a restricted hemagglutination pattern, agglutinating only rat and mouse erythrocytes. Majority of them has receptor-destroying enzyme activities. They are round and enveloped with a diameter of 80 nm. Based on these features and the site of infections, these virus isolates were identified as respiratory bovine coronaviruses (RBCV). The RBCV were previously not recognized to be associated with acute respiratory tract diseases of cattle.

Tests on serum samples from RBCV-positive cattle during a shipping fever epizootic revealed characteristic primary immune responses with specific antibodies to hemagglutinin-esterase and spike. The RBCV-positive cattle that died had only IgM responses to RBCV infections. High level of opsonic and virus-neutralizing IgG2 apparently protected cattle from RBCV infections and respiratory tract disease. These cattle entered this experiment with high antibodies against hemagglutinin-esterase and spike. The G clone cells had maximal susceptibility to RBCV infections from apical

domains. Asymmetric release of RBCV occurred through the apical surfaces of the cells. The RBCV-induced polykaryons had intact plasma membranes and degenerated nuclei, and resulted from expression of spike on cytoplasmic membrane and RBCV replication, indicating fusion from within. The purified RBCV particles showed higher acetylesterase activity at 37°C than at 39°C, while the purified enteropathogenic BCV (EBCV) particles retained full acetylesterase activity at both 37°C and 39°C. Transiently expressed hemagglutinin-esterase of RBCV exhibited a drastic reduction in acetylesterase activity after 40 min at 37°C, while the acetylesterase activity of the transiently expressed hemagglutinin-esterase of EBCV remained stable beyond the 40-min threshold. The deduced amino acid sequences of hemagglutinin-esterase specified by RBCV strains contained specific amino acid changes in comparison to the wild-type EBCV strain, which may be responsible for the observed enzymatic differences.

CHAPTER 1

INTRODUCTION

1.1 Statement of Problem and Hypothesis

We currently observed a newly emerging virus infections among young cattle with respiratory distress on winter pastures in the South, cattle transported to feedlots, and cattle that required veterinary care because they had respiratory tract disease signs during a livestock show in Louisiana (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). These viral isolates were identified as coronaviruses and are referred to as respiratory bovine coronaviruses (RBCV) based on morphological features and cytopathic interaction with the G clone of human rectal tumor-18 (HRT-18) cells. Their unique properties distinguished them from enteropathogenic bovine coronaviruses (EBCV). Distinguishing features are: (1) The RBCV were isolated in the 1st G clone cell passage without the use of trypsin enhancement. Trypsin activation was required for the isolation of EBCV (Storz et al., 1981). (2) The RBCV have unusually high cell-fusing activities for the G clone cells. (3) The RBCV have a restricted hemagglutination pattern. They agglutinate only mouse and rat, but not chicken red blood cells. Logically, questions arise whether RBCV and EBCV are antigenically or genetically different and whether specific tissue tropisms are the basis for such differences. Therefore, the properties of these newly isolated RBCV needed to

be characterized and their pneumopathogenicity had to be defined. Our hypotheses are: (1) The RBCV emerged as an important viral pathogen with control of other respiratory virus infections through effective vaccination and immunization of the cattle population. (2) The RBCV infections were not detected in the past because proper technology was not available. (3) The RBCV developed specific pneumopathogenic properties based on structural and nonstructural genes. (4) The spike (S) and hemagglutinin-esterase (HE) glycoproteins are major determinants in the infectious process.

1.2 Research Objectives

The overall aims of investigations in our research group are to characterize the molecular properties and pneumopathogenicity of RBCV, newly isolated from cattle of this country, and to compare RBCV with EBCV. My specific aims of the research described in this dissertation were: (1) to study the unique properties of the newly emerging RBCV in comparison to EBCV, (2) to identify the antigenic properties of RBCV, and (3) to identify the genetic bases of acetylesterase (AE) activities in the emergence of BCV infecting the respiratory tracts of cattle. My specific research objectives were:

1. To assess the emergence and extend of respiratory coronavirus infections in cattle from 1993 to 1998.

2. To determine the antigenicity of RBCV structural proteins in inducing immune responses of market-stressed cattle during pathogenesis of a severe, naturally occurring shipping fever pneumonia epizootic of 1997.
3. To investigate the susceptibility of polarized epithelioid G clone cells to RBCV and EBCV.
4. To compare the dynamics of cell-fusion induction during the replication of RBCV and EBCV in the G clone cells, and to analyze the RBCV-induced polykaryons.
5. To relate the HE cDNA-predicted amino acid sequences for wild-type strains of RBCV and EBCV to the enzymatic activities of HE glycoproteins of RBCV and EBCV.

Scientific findings of these investigations are presented as 5 individual chapters, most of which represent manuscripts published or submitted for publication to different refereed journals. Their titles are:

1. Dynamics of Emergence of Respiratory Bovine Coronavirus Infections - A Catalogue of Virus Isolates from Major Epizootics.
2. "Antibody Responses to Respiratory Coronavirus Infections of Cattle during Shipping Fever Pathogenesis." Submitted for publication to the "Archives of Virology." Co-authored by K.L. O'Reilly, J. Storz, C.W. Purdy and R.W. Loan.

3. "Infection of Polarized Epithelial Cells with Respiratory and Enteric Tract Bovine Coronaviruses and Release of Virus Progeny." Published by the "American Journal of Veterinary Research" (1997) 58: 1120-1124. Co-authored by K.L. O'Reilly and J. Storz.

4. "Comparison of Fusogenic Properties of Respiratory and Enteric Tract Bovine Coronaviruses." Submitted for publication to the "Journal of Veterinary Medicine." Co-authored by J. Storz.

5. "Hemagglutinin-esterase Specified by Respiratory Bovine Coronaviruses Has Temperature-sensitive Acetylesterase Activity." Submitted for publication to the "Journal of Medical Microbiology." Co-authored by V.N. Chouljenko, K.G. Kousoulas and J. Storz.

CHAPTER 2

LITERATURE REVIEW

2.1 Pathogenic Potential of Coronaviruses

Coronaviruses emerge as increasingly important causes of human and animal diseases. These diseases include respiratory infections, gastroenteritis, hepatic and neurological disorders, immune-mediated disease such as feline infectious peritonitis, and persistent infections (Lai, 1990; Spaan et al., 1988). Bovine coronavirus (BCV) is the second most common cause of virus-induced severe enteritis or occasional pneumoenteritis in calves, and is referred to here as enteropathogenic BCV (EBCV) (Clark et al., 1993; Mebus et al., 1973). Winter dysentery in adult dairy cattle was also attributed to EBCV (Clark et al., 1993, Saif et al., 1988).

2.2 Coronavirus Infections of Cattle

2.2.1 Economic Losses Caused by Bovine Respiratory and Enteric Diseases

The beef and dairy cattle industries remain important economic activities in the United States of America. Over 15,000 family farms and agricultural businesses own almost 1 million cattle as a basis of their livelihood in Louisiana. Nationwide there were over 99 million beef and dairy cattle in 1997 (Weaber, 1998). Respiratory and enteric diseases of cattle represent the most costly diseases in animal agriculture in the U.S.A. It was released by the National Agricultural Statistics Service (NASS), Agricultural

Statistics Board, U.S. Department of Agriculture in May 1996 that a total of 4.38 million head of cattle and calves died from all causes with the total losses at $1.8 billion during 1995 in the United States. Respiratory problems led the other causes with 28% of the total deaths or 1.20 million head. The value of these losses was $478 million. The second leading other cause of deaths in cattle and calves was enteric problems with 20% of the total deaths or 0.86 million head. The value of these losses was $316 million. Coronaviruses are etiological factors in both respiratory and enteric diseases of cattle.

2.2.2 Enteric Diseases of Cattle and EBCV Infections

Severe enteritis (or calf scours) in newborn calves cause an estimated annual loss of over a quarter billion dollars in the U.S.A. and about 1.7 billion dollars worldwide (Ratafia, 1988). Scours and respiratory disease are the major causes of preweaned calf mortality. The EBCV is the second most common cause of viral enteritis in calves (Craig and Kapil, 1994). Although there is geographic variation, EBCV accounts for about 20-26% of the cases of calf scours, and causes acute and chronic diarrhea or pneumoenteritis in young calves (Clark, 1993; Mebus et al., 1973). The EBCV is also involved in winter dysentery in adult dairy cattle (Clark, 1993; Saif et al., 1988).

The primary mechanism of EBCV-induced disease was investigated through findings on ultrastructural features of the interaction of EBCV with the intestinal epithelial cells, early events in EBCV infections of cultured cells, viral protein

processing and cell fusion (Doughri et al., 1976; Storz and Zhang, 1991). The EBCV infection involved the villous epithelial cell lining and some cellular components of the lamina propria mucosae. The EBCV particles interacted with the glycocalyx and adsorbed to the microvilli of intestinal epithelial cells and intercellular membranes of neighboring enterocytes. The adsorbed virions were taken up through fusion of viral envelopes with the plasmalemma of microvilli at the apical surface of intestinal epithelial cells, and by entry into intercellular spaces and interaction with lateral cell membranes. This interaction resulted in alteration of lateral cellular membranes at the site of viral contact. The virus replicated predominantly in the differentiated absorptive epithelial cells and goblet cells of the villi. In the early phase of infection, the rough and smooth elements of the endoplasmic reticulum became distended with electron-dense granulofibrillar material which subsequently accumulated in the apical cytoplasm of infected cells, leading to the formation of viral factories. The viral cores were assembled by condensation of material in cytoplasmic viral factories, and within the distended cisternae of the rough endoplasmic reticulum. Following core condensation, viral envelopment was observed on membranes of cytoplasmic vacuoles, Golgi vesicles, or in association with smooth membrane of viral factories. Release of virus from infected cells occurred either by lysis of the apical plasmalemma and subsequent flow of the cytoplasm with its contents into the gut lumen, by digestion and lysis of extruded

infected cells, or through fusion of virus-containing vacuoles with apical plasmalemma and liberation of their contents.

2.2.3 Respiratory Disease Complex of Cattle and Emergence of RBCV

Bovine respiratory tract disease is recognized as one of the most economically significant concerns of feedlot managers. It is estimated the cattle industry loses $168 to $624 million a year to respiratory disease, with over $112 million of this loss from the feedlot industry (Perino, 1992). The bovine respiratory disease complex (BRDC) represents one of the most costly diseases in the cattle industry (Hoerlein, 1980). Shipping fever pneumonia represents the most serious component of the BRDC, and derives from primary infections with viruses such as bovine herpes virus (BHV-1), bovine parainfluenza virus-3 (PI-3), bovine respiratory syncytial virus (BRSV) or bovine viral diarrhea virus (BVDV) (Ciszewski et al., 1991; Madin et al., 1956; McKercher et al., 1957; Potgieter et al., 1984; Reisinger et al., 1959; Rosenquist et al., 1974). However, secondary bacterial infections lead to deadly pneumonia. Even after years of research and tremendous progress in the development of needed vaccines and antibiotics, BRDC remains a challenging and expensive obstacle to the economically efficient production of cattle. Clearly, there is a continuing evolution of infectious agents and it is hypothesized that new infections may emerge as we achieve control of known virus-induced diseases. The potential of RBCV for causing acute respiratory disease among feedlot cattle was virtually unexpected until the fall of 1993 when Storz

and his colleagues at Louisiana State University (LSU) discovered a high rate of RBCV infections in cattle with respiratory disease on arrival in feedlots (Storz et al., 1996). Extensive investigations at LSU were made on cattle that originated from 11 different states in the U.S.A. during the last 5 years (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). Cattle of weaning age entering the next production system appear highly susceptible to RBCV infections. This surprising finding implies that coronaviruses appear to be a newly emerging factor in the epizootic of shipping fever that was previously not recognized because these viruses did not exist in the past or because they could not be detected by previously employed conventional diagnostic methods. Serological and antigen detection tests identified this type of virus as a potential cause of other forms of respiratory tract disease of cattle (Appel et al., 1992; Carman and Hazlett, 1992; Heckert et al., 1990; Herbst et al., 1989; Jimenez et al., 1989; Möstl and Bürki, 1988). The use of the G clone cell cultures played a cardinal role in the isolation of newly recognized RBCV. Alternatively, successful vaccination programs to prevent known viral infections of cattle may create a shift in favor of this hitherto unrecognized RBCV infections.

2.3 Molecular Properties of Bovine Coronavirus

2.3.1 Coronavirus Taxonomy

Coronaviruses, a genus in the family *Coronaviridae*, are large, enveloped positive-strand RNA viruses that are important causes of human and animal diseases (Lai, 1990;

Spann et al., 1988; Wege et al., 1982). The viruses have the largest genomes of all RNA viruses. Three serologically distinct groups of coronaviruses have been identified. Bovine coronavirus belongs to the antigenic group II. Recently, toroviruses were classified as a genus within the *Coronaviridae*, and arteriviruses were also proposed as a new genus in this family (Cavanagh and Horzinek, 1993; den Boon et al., 1991; Snijder et al., 1991). Later, the International Committee on Taxonomy of Viruses in Jerusalem Meeting in 1996 created a new family, the family *Arteriviridae*, which contains a single genus arterivirus.

2.3.2 Structure of the BCV Virion

Bovine coronavirus is a round, enveloped particle with a diameter of around 80 nm (Lai, 1990) (Fig. 2.1). The viral envelopes are formed by budding from intracellular membranes, and covered with a distinctive fringe of 20 nm long petal-shaped surface projections. The viral genome, is a single-stranded, positive-sense, RNA molecule with an estimated molecular weight of 8×10^6 dalton (Da) or 31 kilobases (kb). The viral RNA genome is associated with the nucleocapsid phosphoprotein, N, to form a long, flexible, helical nucleocapsid. There are 4 structural proteins on its lipoprotein envelope: (1) membrane glycoprotein (M, formerly called E1), (2) spike glycoprotein (S, formerly called E2), (3) hemagglutinin-esterase glycoprotein (HE, formerly called E3), and (4) recently identified envelope protein (E).

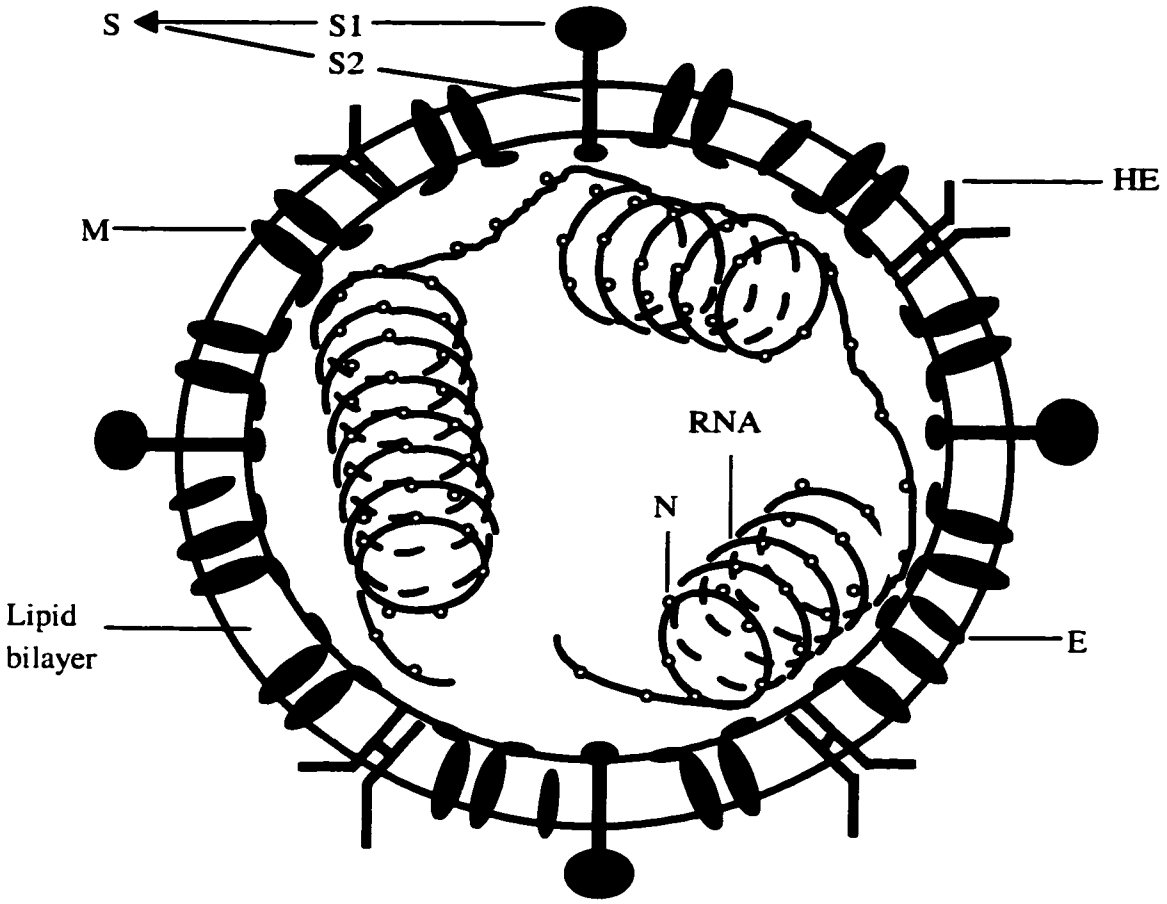


FIG. 2.1: Model of coronavirus structure.

2.3.3 Viral Genome Structure and Organization

The 31kb RNA genome of BCV is capped, polyadenylated, and can function as mRNAs (infectious) (Lai, 1990). The overall location and order of the known genes of BCV and other hemagglutinating (HA) coronaviruses is: 5'-RNA pol-N_s (nonstructual) 32-HE-S-N_s 4.9-N_s4.8-N_s12.7-E-M-N-poly (A)-3' (Fig. 2.2). The first gene at the 5' end of the genome is roughly 21 kb long and represents two-thirds of the entire genome (Lai, 1990, Spann et al., 1988). This large gene region consists of 2 overlapping open reading frames (ORFs, ORF1a and ORF1b) that encode the viral RNA-dependent RNA polymerase, proteases, and other as yet uncharacterized proteins. However, the structure of this gene has not been determined. The terminal 3' end of the genomic RNA consists of approximately 9.5 kb and contains the genes for 5 major structural proteins (S, HE, E, M, N) and at least 4 other smaller proteins (N_s32, N_s4.9, N_s4.8, N_s12.7) which may play structural and nonstructural roles (Abraham et al., 1990, Cavanagh et al., 1990; Spaan et al, 1988).

2.3.4 Structures and Functions of Structural and Nonstructural Proteins

Nucleocapsid protein: The BCV N protein is an internal non-glycosylated 50-52 kDa protein with a backbone of 448 amino acids (a.a.) and specific phosphorylation sites on serine residues, and it is rich in clusters of basic residues within the middle RNA-binding domains (Laude and Masters, 1995). This protein binds to virion RNA

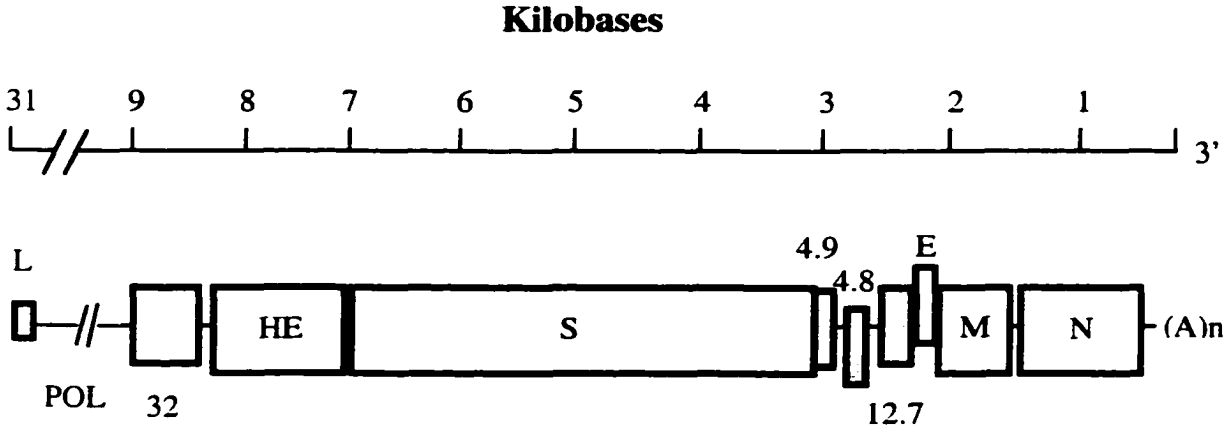


FIG. 2.2: Organization of eneteropathogenic bovine coronavirus genome.

HE gene:	1272 n.t.	→ Hemagglutinin-esterase (HE) gp of 424 a.a.
S gene:	4092 n.t.	→ Spike (S) gp of 1363 a.a.
M gene:	690 n.t.	→ Matrix (M) gp of 230 a.a.
N gene:	1344 n.t.	→ Nucleocapsid (N) protein of 448 a.a.
E gene:	252 n.t.	→ Small envelope (E) protein of 84 a.a.
32-KDa ORF:	831 n.t.	→ Ns 32KDa-protein of 277 a.a.
4.9-KDa ORF:	129 n.t.	→ Ns 4.9KDa-protein of 43 a.a.
4.8-KDa ORF:	135 n.t.	→ Ns 4.8KDa-protein of 45 a.a.
12.7-KDa ORF:	327 n.t.	→ Ns 12.7KDa-protein of 109 a.a
L:	Leader sequence	
POL:	RNA polymerase	

genome, providing the structural basis for the long, flexible, helically symmetric nucleocapsid. Its major activity is involved in the process of viral RNA replication.

Membrane protein: The 26-32 kDa M glycoprotein contains 230 a.a., is derived from the 22 kDa protein backbone with 1 or 2 oligosaccharide side chains (2 kDa), and exists as a set of different protein species due to different levels of glycosylation (Rottier, 1995). It is the most abundant viral structural protein with a short N-terminal domain exposed on the exterior of the viral envelope, 3 hydrophobic α-helices spanning the lipid bilayer 3 times, and a large C-terminal domain located in the interior of the virus particle. The M protein of BCV is targeted to Golgi apparatus and does not appear on the plasma membrane (Machamer et al., 1990; Machamer and Rose, 1987; Swift and Machamer, 1991). This protein is essential for envelope formation, appears to play a key role in binding the helical nucleocapsid to the viral envelope during virus budding, and determining viral budding site on intracellular membranes. Monoclonal antibodies specific for the M protein of coronavirus do not neutralize virus infectivity. The M protein of coronaviruses is very closely related among virus strains of the same antigenic group, but differs largely from all the others (Rottier, 1995).

Hemagglutinin-esterase protein: The HE glycoprotein consists of 424 a.a. with an N-terminal signal region, a C-terminal anchorage region, and N-linked glycosylation sites, and is composed of 2 identical, disulfide-linked 65 kDa subunits (Deregt et al., 1987; King et al., 1985). This glycoprotein is found only in the envelopes of certain

species of coronviruses in antigenic group II, including BCV, porcine hemagglutinating encephalomyelitis virus (HEV), human respiratory coronavirus (HCV-OC43), turkey coronavirus (TCV) and some strains of mouse hepatitis virus (MHV). It forms short spikes on the viral envelope with molecular mass of 65 kDa and 140 kDa in its reduced and nonreduced forms, respectively. The bovine coronavirus HE genes have a.a. sequences homology with HE glycoprotein of influenza C virus and may have been derived by a recombination between an HE mRNA of influenza C virus and the genomic RNA of an ancestral coronavirus (Luytjes et al., 1988). The BCV HE has both hemagglutinating, receptor-binding and AE-mediated receptor-destroying functions which are similar to the influenza C virus. The HE glycoprotein of BCV binds to the 9-O-acetylated neuraminic acid residues of glycoproteins or glycolipids on the surfaces of erythrocytes and susceptible cells, which is considered to be the major receptor determinant of coronavirus (Herrler et al., 1985; Herrler et al., 1991; Vlasak et al., 1988a). The AE activity of HE hydrolyzes an ester bond to cleave an acetyl group from the 9-O-position of the substrates, potentially eluting adsorbed virions and destroying the bond between HE and the glycans on the cell membrane. Inhibition of the AE activity of BCV HE by diisopropylfluorophosphate resulted in remarkable reduction of viral activity, indicating that binding of HE to 9-O-acetylated neuraminic acid residues can facilitate virus infection (Vlasak et al., 1988a). Enzymatic removal of the 9-O-acetylated neuraminic acid from cell membranes or treatment with HE-specific

monoclonal antibodies inhibits BCV infections (Deregt et al., 1989; Schultze and Herrler, 1992). Furthermore, cells infected with a baculovirus recombinant expressing the BCV HE exhibited hemadsorption and esterase activities, both of which could be blocked by monoclonal antibodies which have infectivity neutralization activity (Parker et al., 1990a; Yoo et al., 1992).

Spike protein: The S protein of BCV is N-link glycosylated with a molecular mass of 185-190 kDa, and forms the longer peplomeric projections on the virion envelope (Cavanagh, 1990). This glycoprotein contains 1363 a.a. with an N-terminal signal sequence, a coil-to-coil structure, and a C-terminal hydrophobic membrane-anchoring domain. The S precursor is frequently cleaved into 2 functional subunits in BCV and some strains of MHV: the N-terminal 110 kDa S1 and the C-terminal 100 kDa S2. Proteolytic cleavage of S preprotein is considered to enhance cell-fusing activity and/or plaque-forming activity for some host cells, but it is not absolutely required for induction of cell fusion for some MHV strains or mutants, feline infectious peritonitis virus (FIPV) and transmissible gastroenteritis virus (TGEV) which lack the protease motif (Cavanagh et al., 1990; St. Cyr-Coats et al., 1988b; Storz et al., 1981; Sturman et al., 1985). This protein of BCV is responsible for virus binding to 9-O-acetylated neuraminic acid-containing glycans on the target cells, initiation of infection, induction of cell-to-cell fusion after cell-surface expression (fusion from within), elicitation of neutralizing antibodies and cell-mediated immunity (Spaan et al., 1988). Furthermore,

purified S protein was documented to exhibit higher hemagglutinating activities with rodent erythrocytes than purified HE protein (Schultze et al., 1991a and b). Although both S and HE glycoproteins of the BCV L9 strain were able to agglutinate rodent erythrocytes, only S could agglutinate chicken erythrocytes (Schultze et al., 1991a). The 9-O-acetylated neuraminic acid was identified as receptor determinant on susceptible cells for BCV to initiate infection, and was bound by both HE and S viral proteins. However, S glycoprotein of BCV was considered to be the major viral structural protein to bind to neuraminic acid-containing glycans (Schultze et al., 1991b). A porcine respiratory coronavirus was found with a deletion in the S gene region when compared with the TGEV (Rasschaert et al., 1990). This deletion was most likely responsible for the differences in tissue-tropism and pathogenicity of the 2 viral strains. Recently, 2 a.a. changes at the N-terminus of the TGEV S glycoprotein were reported to result in the loss of enteric tropism, which further suggested that the S1 subunit is directly involved in the enteropathogenic potential of TGEV (Ballesteros et al., 1997). Further investigations of TGEV S1 subunit with point mutations or a deletion of 4 a.a. within residues 145 to 155 lead to reduced enteropathogenicity and loss of hemagglutinating activity (Krempl et al., 1997)

Envelope protein: A small (9- to 12-kDa) integral hydrophobic protein E was recently identified in the viral envelope of BCV, and is encoded by a 9.5 kDa ORF which was previously believed to be a nonstructural gene, (Brown and Brierly, 1995).

This protein was found in small amounts in infected cells and in the virion particles with its unknown function.

Nonstructural proteins: The 32 kDa nonstructural protein was documented to be a phosphoprotein that accumulates in the cytoplasm of infected cells (Cox et al., 1989; Cox et al., 1991). Between the S and M genes within the BCV genome, there are 3 different ORFs encoding nonstructural proteins of 4.9, 4.8 and 12.7 kDa (Abraham et al., 1990a). The overall transcriptional pattern of this region is remarkably different between BCV and MHV (Lai, 1990). The 4.9 kDa ORF, most likely, is not translatable, while no information is available whether the 12.7 kDa and 4.8 kDa ORFs are expressed in infected cells (Hofmann et al., 1993). The functions of these gene products are unknown. It is also uncertain whether they play a role in virus pathogenesis.

2.3.5 Coronavirus Replication

Like other positive-strand RNA viruses, BCV does not contain any RNA-dependent RNA polymerase in their virions. The virus replicates in cytoplasm of infected cells by a unique mechanism that results in a high frequency of recombination (Lai, 1990) (Fig. 2.3).

Attachment and penetration: Upon infection, the BCV binds to the plasma membrane of target cells by the interaction of S glycoprotein with its specific receptor glycoprotein, 9-O-acetylated neuraminic acid moieties on membrane macromolecules

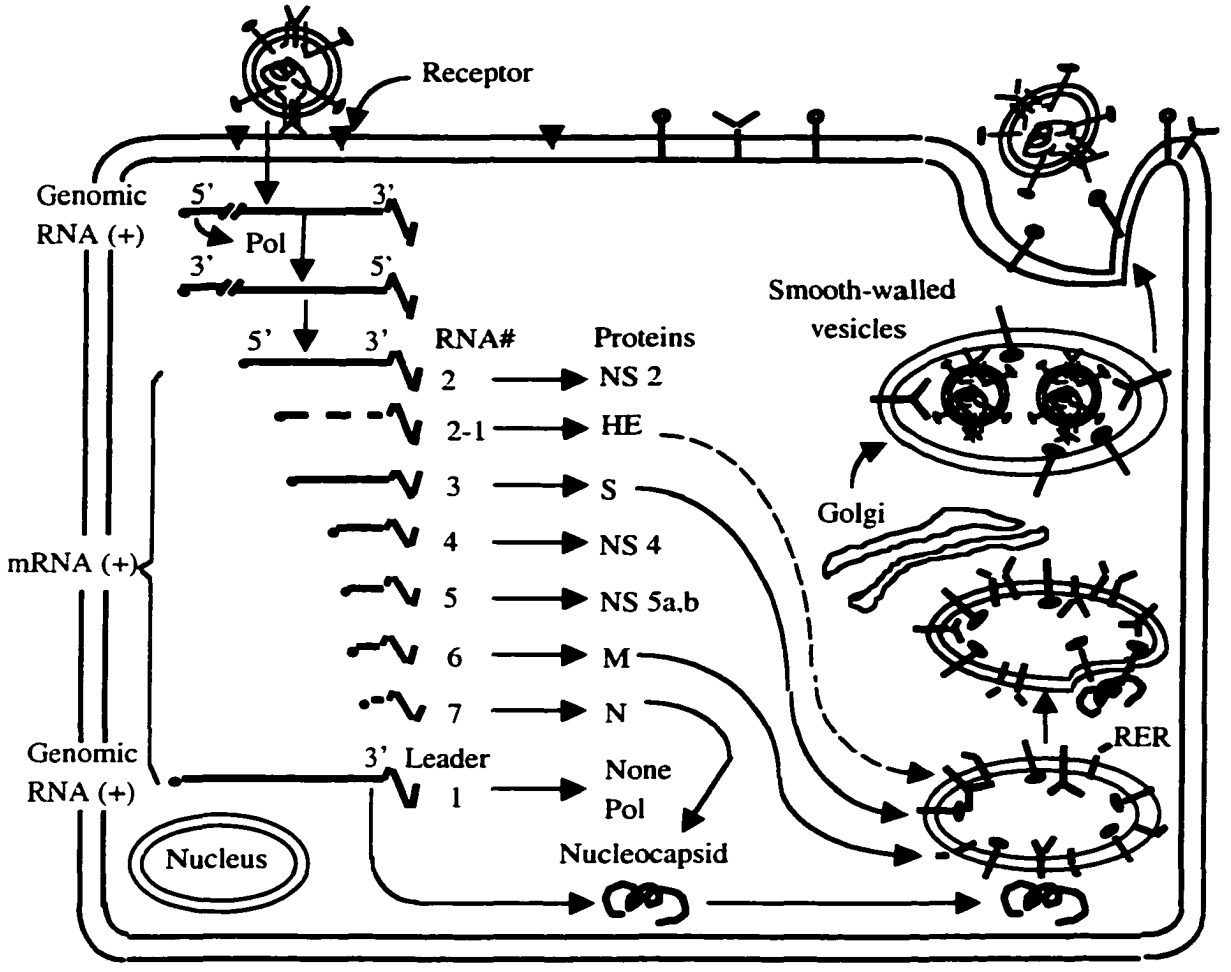


FIG. 2.3: Model of coronavirus replication.

(Schultze et al., 1991a and b; Schultze and Herrler, 1992). Binding of HE glycoprotein in the short peplomeres to 9-O-acetylated neuraminic acid residues on the cell surface may function as a second viral attachment protein besides the S protein. The virus penetrates through fusion of the viral envelope with the plasma membrane that is mediated by the S2 subunit of S protein.

Primary translation: Following entry of viral genome into the cytoplasm, the positive-strand genomic RNA is first translated to form a macromolecule (>800 kDa) which is co- or posttranslationally processed by viral and cellular proteases to produce multiple proteins that serve as a virus-specific, RNA-dependent RNA polymerase. Although p28, p290, p50, p240 and several other cleavage products were found in MHV-infected cells, the primary gene product has not been clearly identified (Baker et al., 1989; Baker et al., 1993; Denison et al., 1991; Denison et al., 1992; Denison and Perlman, 1986; Denison and Perlman, 1987)

Transcription of viral RNA: The polymerase then transcribes positive-strand genomic RNA into a full-length negative-strand RNA, which is subsequently used as a template for synthesis of new positive-strand viral genomic RNA and mRNAs which are capped and polyadenylated (Sawicki and Sawicki, 1990).

The RNA polymerase must recognize specific signals that control the initiation of positive- and negative-stranded RNA synthesis. Deletion mapping of different defective

interfering RNAs (DIs) demonstrated that sequences comprising both the 3'-terminal and the 5'-terminal of viral genomes are required for viral replication, but the sizes of these regions were different for different DIs (Van der Most and Spaan, 1995).

The mRNA: All the mRNAs are co-nested on the 3' ends. Although each of the mRNAs except the smallest is polycistronic, only the unique 5'-end ORF of each mRNA, which is not present in the next smaller mRNA, is translated into a protein (Leibowitz et al., 1982; Siddell, 1983). Subgenomic negative-strand RNAs are also synthesized in double-stranded forms during viral replication (Sawicki and Sawicki, 1990; Sethna et al., 1989). Both subgenomic and genomic-length negative-strand RNAs serve as templates for subgenomic mRNA synthesis. Eight virus-specific mRNAs were detected in cells infected with bovine coronavirus (BCV) and other hemagglutinating (HA) coronaviruses indicating the existence of 8 subgenomic mRNAs.

The subgenomic mRNAs of coronaviruses not only form a 3'-co-terminal nested set but also are characteristically 5'-co-terminal. All subgenomic mRNAs of BCV contain a common 5' end of a leader sequence about 65 nucleotides (n.t.) long which is encoded by the 3' end of the negative-strand template and derived from the 5' end of the genomic RNA (Hofmann et al., 1993). This leader sequence is not found elsewhere in the genome, but between each ORF on the genome there is a short intergenic sequence (IS) that contains a 5-11 n.t. sequence homologous with a sequence near the 3' end of

the BCV leader RNA (Hofmann et al., 1993; Shieh et al., 1987). It is not clear how the 5' terminally encoded leader recombines with the 3' co-terminal subgenomic mRNAs.

Models for coronavirus mRNA synthesis: There are 3 models for coronavirus mRNA synthesis. Leader-primed transcriptional mechanism is a model which is most popularly accepted (Lai, 1990) (Fig. 2.4A). Transcription of leader RNA would begin at the 3' end of the full-length, negative-strand template RNA and terminate with the dissociation of the leader from the template, either alone or with attached polymerase protein(s). The leader would then recognizes its complementary promoter at ISs downstream on the negative-strand template and function as the primer for mRNA transcription. The leader sequences can function *in cis* and *in trans* and can serve as primers for positive-stranded RNA synthesis (Lai, 1990). In this model, the ISs serve as promoters for mRNA transcription. A second model for coronavirus RNA synthesis is called discontinuous transcription mechanism (Sawicki and Sawicki, 1990) (Fig. 2.4B). During negative-strand RNA synthesis, the polymerase would stop at one of the ISs and then jump to the 3' end of the leader sequence near the 5' end of the genomic RNA template, generating subgenomic negative-strand RNA with an antisense leader sequence at its 3' end. These subgenomic negative-strand RNAs and a full-length negative strand could serve as templates for continuous transcription of positive-strand mRNAs and genomic RNA. In this model, the IS functions as transcriptional termination sequence and/or as a sequence that interacts with leader RNA to permit

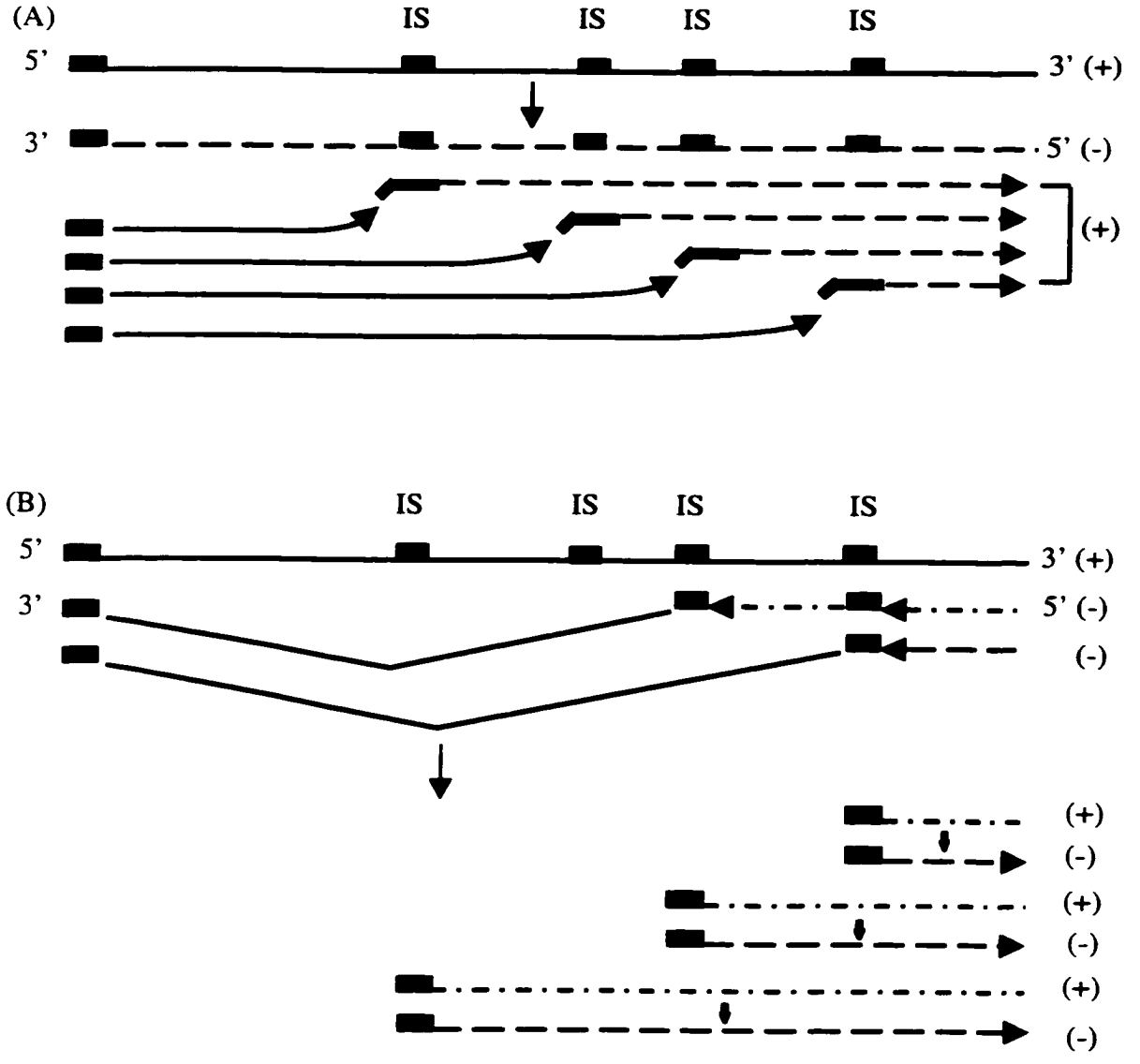


FIG. 2.4: Models for transcription of coronavirus subgenomic mRNAs.

(A) Leader-primed transcription during (+) strand synthesis, (B) Discontinuous transcription during (-) strand synthesis.

polymerase jumping during negative-strand RNA synthesis. A third model proposes that, following the penetration of virus, the positive-strand subgenomic mRNAs are used directly as templates for transcription of the negative-strand subgenomic RNAs, which are subsequently used as templates for synthesis of additional subgenomic mRNAs (Sethna et al., 1989; Sethna et al., 1991).

Replication of viral genomic RNA: Production of full-length, positive-strand genomic RNA requires continuous synthesis with the full-length, negative-strand template. Studies of cloned DI RNA of MHV virus indicated that around 400 n.t. at both the 3' and 5' ends of viral genome, and an internal sequence of about 135 n.t. were required for replication of DI RNA (Kim et al., 1993; Lin and Lai, 1993; Masters et al., 1994). Furthermore, a free leader sequence of the DI RNA was suggested to be involved in RNA replication (Makino and Lai, 1989). However, the precise mechanism of viral RNA replication remains unknown.

Translation, processing and intracellular transport of viral proteins: Huge amounts of N protein are translated on free polysomes from mRNA 7, and rapidly phosphorylated in the cytosol (Stohlman et al., 1983). The HE, S, and M glycoproteins are encoded by mRNAs 2-1, 3, and 6, respectively, and translated on membrane-bound polysomes on the rough endoplasmic reticulum (Sturman and Holmes, 1983). The S glycoprotein is cotranslationally inserted into the RER and glycosylated at asparagine residues (N-linked), oligomerized into noncovalently linked homotrimers, and

transported through the Golgi apparatus, where the S2 subunit is fatty-acylated, and the high mannose oligosaccharides are trimmed with addition of terminal sugars (Delmas and Haude, 1990; Opstelten et al., 1993; Schmidt, 1982; Sturman et al., 1985). The S protein may be proteolytically cleaved to form two 100-110 kDa subunits (Abraham et al., 1990b; Deregt et al., 1987). Excess S protein that is not incorporated into virions is transported to the plasma membrane, where it plays a role in cell-cell fusion (Griffiths and Rottier, 1992; Vennema et al., 1990). The matrix glycoprotein M is transported to the Golgi apparatus, where it accumulates and may be glycosylated at serine or threonine residues (O-linked), but M is not transported to the plasma membrane (Locker et al., 1992). The HE protein is also cotranslationally glycosylated at asparagine residues (N-linked) in the RER. Subsequently the processed HE is transported to the Golgi when the N-linked glycans are removed (Yokomori et al., 1989), and then to the plasma membrane, where it can cause hemadsorption (Kienzel et al., 1990).

Assembly and release of virions: The newly formed positive-strand genomic RNAs and N proteins assemble in the cytoplasm to form helical nucleocapsids (Sturman and Holmes, 1983). The N protein binds to RNA in both sequence- and non-sequence-specific manners with a preferred binding site within the leader RNA (Robbins et al., 1986; Stohlman et al., 1988). Packaging of genomic RNA needs a specific signal in genomic RNA which consists of a stretch of 61 nt in the 3' end of gene 1, approximately 20kb from the 5' end of the genome (Fosmire et al., 1992; van der Most

et al., 1991). Virions mature by budding of nucleocapsids from the membranes of the Golgi apparatus or endoplasmic reticulum that contain M protein (Dubois-Dalcq et al., 1982; Tooze et al., 1984; Tooze and Tooze, 1985). The unique large and small peplomeres formed by S and HE, respectively, are incorporated into virions at the time of budding. It is still unknown whether Golgi-associated posttranslational processing of viral structural glycoproteins occurs before or after their incorporation into virions.

Following budding, virions accumulate in large, smooth-walled vesicles and are released from the cell cytoplasm presumably by fusion of the virion-containing vesicles with the plasma membrane (Griffiths and Rottier, 1992).

CHAPTER 3

DYNAMICS OF EMERGENCE OF RESPIRATORY BOVINE CORONAVIRUS INFECTIONS-A CATALOGUE OF VIRUS ISOLATES FROM MAJOR EPIZOOTICS

3.1 Introduction

Bovine respiratory and enteric tract diseases represent the most costly diseases in the cattle industry of the United States. The role of coronavirus in causing severe diarrhea in neonatal calves was initially explored in the seventies (Doughri et al., 1976; Mebus et al., 1973). Coronavirus might also be involved in winter dysentery of adult cattle as reported by Saif and co-workers in 1988. Mebus and co-workers (1973) succeeded in adapting the enteropathogenic coronavirus strains L9 (EBCV-L9) through inoculation of a diarrhea specimen into a specific culture of bovine fetal kidney cells through multiple passages. Adaptation of other wild-type EBCV agent to cultured bovine cells remained unsuccessful in numerous instances in which electron microscopic examination revealed the presence of intestinal coronavirus infections. However, wild-type EBCV strains could be isolated and propagated in the HRT-18 cell line, which was derived from human rectal adenocarcinoma (Laporte et al., 1979; Tompkins et al., 1974). This process was enhanced by addition of trypsin to the culture medium (St. Cyr-Coats et al., 1988a). The EBCV are among the better characterized hemagglutinating coronaviruses which also include human coronavirus strain OC43,

hemagglutinating encephalomyelitis virus of swine, the turkey coronavirus, and mouse hepatitis virus strain JHM (Spaan et al., 1988; Storz et al., 1992).

Shipping fever pneumonia is an acute respiratory tract disease, and remains a serious problem in beef cattle recently transported to feedlots. This disease is commonly considered to be induced by multiple factors whereby stressful conditions, such as transport and assembly, favor primary respiratory tract infections with viruses which become further complicated by secondary bacterial infections with *Pasteurella (P.) haemolytica* or other bacterial species (Hoerlein, 1980; Yates, 1982). Viruses traditionally associated with shipping fever pneumonia include BHV-1 (McKercher et al., 1957), bovine PI-3 virus (Baldwin et al., 1967; Reisinger et al., 1959), bovine pneumovirus or BRSV (Rosenquist, 1974) and pestivirus of BVDV (Potgieter et al., 1984). Bovine herpesvirus-1 was isolated by Jensen and co-workers (1976) from respiratory tract samples in 18% of 354 cases of shipping fever pneumonia among cattle in Colorado feed yards. However, dynamics of any of these virus infections have not been explored in naturally evolving shipping fever epizootics (Yates, 1982).

European reports on serological and epidemiological investigation indicate that coronaviruses may play an etiological role in respiratory tract disease of young calves (Herbst et al., 1989; Jiminez et al., 1989; McNulty et al., 1984; Möstl and Bürki, 1988). Little information exists on the prevalence and epidemiology of respiratory coronavirus infections in adult cattle of the United States (Carman and Hazlett, 1992; Heckert et al.,

1990). However, all these investigations provided serologic and other indirect evidence for respiratory coronavirus infection in cattle. The wild-type RBCV was not isolated previously from feedlot or other adult cattle by conventional cell culture techniques. Therefore, RBCV have neither been characterized nor compared with the known EBCV because reliable isolates were not available.

In 1993, one investigation was conducted in the U.S.A. to assess prevailing viral respiratory infections of cattle (Storz et al., 1996). This experiment involved 100 cattle in Kansas and Arizona feedlots. A high percentage of cattle arriving at feedlots yielded isolates of an "emerging" RBCV. These viruses were recovered from nasal swab samples collected from cattle experiencing acute respiratory distress following shipping. A novel isolation scheme was employed which included the G clone cells and 2 cell types of cultured bovine cells with specific ranges of permissiveness for all known respiratory viruses of cattle including RBCV. This approach resulted in the first successful isolation of RBCV at high rates from nasal swab samples of cattle arriving at feed yards with respiratory distress, and provided the first evidence for an etiological role of this virus in naturally occurring shipping fever pneumonia. This virus was also isolated at high rates from young cattle of a livestock show and stocker cattle on pastures during fall and winter months in the southern United States (Storz, 1998; Storz et al., 1999).

During the fall of 1997 and 1998, 2 prospectively designed investigation were conducted on 105 and 120 cattle, respectively, which experienced severe epizootics of naturally occurring shipping fever pneumonia and were subjected to sequential sampling and bacteriological, virological and serological testing (Storz et al., 1999, Storz et al., 2000a and b). These experiments generated excellent samples for virological studies on naturally-infected cattle. Again, a surprisingly high rate of RBCV infections and virtually no other bovine respiratory viruses were detected from nasal swab and lung samples of sick or dead cattle at the beginning of these shipping fever epizootics.

The epizootic dimensions of this RBCV infection of beef and dairy cattle are documented by isolation of this newly emerging virus from cattle with acute respiratory tract diseases in 11 different states of this country. This finding implies that RBCV appears to be a new factor in the BRDC that was previously not recognized. It is possible that this virus infection was missed in past investigations because sensitive tests were not applied, but a more likely hypothesis is that we are dealing with an emerging virus infection in a cattle population that is protected through effective modern vaccination against other known virus infections. The major objectives of this investigation were to assess the dynamics of currently prevailing respiratory virus infections of cattle with acute respiratory tract diseases, including shipping fever pneumonia, and to catalogue virus isolates with features of cytopathic expression of

RBCV in G clone cells, and to identify hemagglutinating and receptor-destroying properties.

3.2 Materials and Methods

3.2.1 Experimental Design and Collection of Samples from Cattle

1993 Epizootic: One hundred cattle of 6 to 9 months old were involved in the 1993 experiment. Fifty beef cattle had originated from farms and ranches in Oklahoma, Texas and Arkansas, has passed through sale yards, then were transported to a feedlot in Kansas. Another 50 young cattle originated from dairy herds in California and were transported to a feedlot in Arizona. These cattle showed respiratory signs and fever on arrival at the 2 feedlots when clinical examinations were performed and nasal swab samples were taken. However, ensuing respiratory tract diseases of these cattle was not monitored. Duplicate nasal swab samples were obtained and placed in separate tubes containing 3 ml of transport medium (phosphate buffered saline [PBS] with 5% fetal calf serum), and were then frozen at –70°C until they were tested. One set of these samples was tested at our laboratories in Louisiana State University while another set was assayed at a laboratory in Kansas.

Cases from Louisiana State University livestock show and clinics: Twenty-nine nasal swab samples were obtained from cattle in the 1994 Louisiana State University Livestock Show (LSU-LSS). These calves showed signs of acute respiratory distress and required special veterinary services. From 1995 to 1997, 16 nasal swab samples

were supplied from clinical cases of LSU Department of Veterinary Clinical Sciences and Veterinary Medical Diagnostic Laboratory and from clinical cases of Mississippi State University Veterinary Clinical Sciences.

Cases from cattle on winter pastures: Fifteen nasal swab samples were obtained on November 25, 1996 from cattle of the Lawton Ranch Herd which had acute respiratory tract disease and were provided by Dr. Michael Meaux at Rhode Veterinary Hospital, Inc. These cattle presented with mucopurulent nasal discharge and temperature of 41°C. Lung sounds were greatly increased with crackles and wheezes present. The animals were treated with Micotil. The herd consisted of 900 head of purebred Charolais cattle which originated from Texas, and were divided into several pastures located just west of Sulphur, Louisiana. This herd was closed with the only new additions to the herd being Charolais, Limosine and Beefmaster bulls and 3 Charolais cows with calves a year and a half earlier. At weaning, these cattle subcutaneously were given a vaccine containing killed IBR, BVD, PI-3, BRSV virus antigens as well as a 5-way lepto and a 8-way clostridium vaccines. They were dewormed and heifers were brucellosis vaccinated.

One set of trachea and lung samples of 3 dead calves was provided by Dr. Jeffrey Anderson from a farm in the Northwest Louisiana during March of 1998. This 3-month old calf was from a herd of 150 beef cows, 125 calves, and 23 cow-calf pairs which experienced a severe respiratory disease episode with over 20% of the 125 calves

affected. The affected calves had pneumonia with mucopurulent nasal discharge, increased respiratory rates, and rectal temperature above 40°C. They had been vaccinated with "Cattlemaster 4+VL5 (BHV-1, BVDV, PI-3, BRSV, leptospira and campylobacter antigens), 1 shot of *Pasteurella* vaccine, and Scour-guard K/C (coronavirus, rotavirus, and *Escherichia coli*) about 2 weeks before the onset of pneumonia epizootic. In November, 1998, another 2 sets of trachea and lung samples from 2 dead calves were provided by Dr. Anderson. The calves were 6 months old and from a herd of 200 cows and 200 yearling cattle. Clinical features and vaccine histories were the same as described before. All trachea and lung samples were frozen at –70°C until they were tested.

1997 and 1998 epizootics of shipping fever among market-stressed cattle: Prospective, carefully designed experiments of 1997 and 1998 included 105 and 120 cattle of 6 to 8 months old, respectively. The cattle were assembled at an order-buyer barn (OBB) from 3 different local auctions in eastern Tennessee. Each calf was eartag-identified, and clinically examined on their arrival at the OBB, that is day 0 post-arrival at OBB. After nasal swab and blood samples had been collected, the cattle were vaccinated with commercially available modified-live vaccines against BHV-1 and PI-3 in 1997 (Prevail, Rhone Merieux, Inc., Athens, Ga.) and BHV-1 only in 1998 (Reliant, Rhone Merieux, Inc., Athens, Ga.). A 7-way clostridial vaccine (Electroid 7, Mallinckrodt Veterinary, Inc., Mundelein, Il.) and Ivomec (Merck Agriculture and

Veterinary Division, Merck and Co., Inc., Rahway, N.J.) were given to all cattle and an experimental bacterin-toxoid of *P. haemolytica* was given to the odd-numbered cattle (Loan et al., 1988). After a 4-day rest at the OBB, the cattle were transported 1932 kilometers to a feed yard jointly operated by the Agricultural Research Service (ARS) and the Texas Agricultural Experiment Station in Bushland, Texas where clinical assessments were continued. Nasal specimens were taken on days 7, 14, and 21 post-arrival at OBB, while blood specimens were taken on days 7, 14, 21, 28 and 35 post-arrival at OBB. Nasal swab samples were collected in duplicates using cotton swabs that were inserted deeply into the ventral meatus of the nostrils, placed into test tubes with 1 ml transport medium, and frozen at –70°C until they were subjected to virological and bacteriological tests while blood samples were obtained for serum harvest.

Ten and 16 cattle died during the 1997 and 1998 investigations, respectively. The dead cattle were taken to the Texas Veterinary Diagnostic Laboratory in Amarillo, Texas, where necropsies were performed and lung samples were collected. Aliquots of lung samples were frozen at –70°C for isolation, identification and quantitation of bacteria and viruses.

3.2.2 Processing of Nasal Swab Samples

The samples were thawed, and 1 ml of cold Dulbecco's modified minimal essential medium (DMEM, Sigma Chemical Co., St. Louis, Mo.) with 4.5 g/l of glucose buffered

with 44 mM $NaHCO_3$ and supplemented with penicillin (100 units/ml) - streptomycin (100 µg/ml) (Sigma Chemical Co.) was added to each sample. The tubes were stirred for 2 min. Aliquots of fluids were taken for centrifugation at 1,500 × g for 20 min. Supernatant fluids were withdrawn and filtered through 0.45 µm Millipore filters (Acrodisc™ syringe filters, Gelman Sciences, Ann Arbor, Mich.). The filtrates were used to inoculate the cell cultures.

3.2.3 Processing of Trachea and Lung Samples

Virus isolation and titration: Tracheal mucosa and lung tissues were cut from frozen specimens and minced with scissors into small pieces, while surface mucus membranes of trachea were scrapped off with scissors. Tissues were homogenized by grinding with Ten Broeck devices in the appropriate volume of cold DMEM to make 5% (g/v) suspensions. The homogenates were centrifuged at 1,500 × g for 20 min. The top half of the supernatant was withdrawn, diluted 1:1 with cold DMEM and subjected to a second cycle of centrifugation. Then, the top half of this supernatant was withdrawn, and filtered as described and used for virus isolation and RBCV infectivity titration.

Immunocytochemical examinations: A small piece of lung tissue was cut from each thawed sample and used to make exfoliative cytological preparations on glass slides for immunofluoresecent tests.

Bacterial identification and quantitation: Tests were performed according to Weaver and Frank (Frank and Wessman, 1978; Weaver and Hollins, 1980). Briefly, the lung specimens were thawed, surfaces were seared with a hot spatula, and 1 gram of tissue was removed from the center of each sample. The cut tissue was minced with scissors, homogenized with a Ten Broeck device, and subjected to a serial 10-fold dilution in PBS. Aliquots of 0.1 ml of the dilutions were plated in duplicate on tryptose agar plates fortified with 5% citrated bovine blood, and spread over the media with a spreader bar. The culture plates were kept in a 37°C incubator with 5% CO_2 for 24 h. Bacterial colonies were counted and the numbers of the 2 replicates were averaged. Colonies of *P. haemolytica* and *P. multocida* were identified by colony morphology, Gram staining, biochemical reactions and specific serotyping.

3.2.4 Virus Isolation Scheme for Known Respiratory Bovine Viruses

The virus isolation scheme included 3 different cell types: the G clone of human rectal tumor-18 (HRT-18) (Lin et al., 1997b; Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b; Tompkins et al., 1974), Georgia bovine kidney (GBK) and bovine turbinate (BT) cells. These cell types were specifically permissive for all known respiratory bovine viruses. The BT cells are particularly permissive for BRSV (Rosenquist, 1974; Storz et al., 1996), while either BT or GBK cells are sensitive to other viruses, such as PI-3, BHV-1, cytopathogenic BVDV and bovine adenovirus (Storz et al., 1996). Importantly, RBCV can only replicate in the G clone cells and not

in currently available cultured bovine cells (Storz et al., 1996). Virus replication and related cytopathic changes in these cell culture systems were tested with the Cooper strain of BHV-1, the 72-P-1096 strain of PI-3, the NADL strain of BVDV, the FS1-1 strain of BRSV, and the LSU-LSS-218 strain of bovine adenovirus.

Different cell types were propagated in sets of 24-well plates (Becton-Dickinson Labware, Franklin Lakes, N.J.) with DMEM at 37°C until confluent monolayers were formed. Medium supplemented with 5% fetal bovine serum (FBS, Sigma Chemical Co.) was used for cell propagation, and serum-free medium was used during virus propagation. Confluent monolayers were washed 2 times with serum-free Dulbecco's PBS (calcium and magnesium free, Sigma Chemical Co.) before inocula were added. Each prepared sample was diluted to 10^{-1} and 10^{-2}, and 0.5 ml of each dilution was added to each well in duplicates. Five samples were tested to 5 rows of 4 wells of each plate, leaving the 6th row of 4 wells as uninoculated cell control. Following inoculation, the cell cultures were incubated at 37°C and examined daily with an inverted microscope for evidence of cytopathic changes for 6 days. Then, they were subjected to 1 cycle of freezing at –70°C and thawing at room temperature. The media of cell cultures in the 4 wells inoculated with the same test samples or the control wells were pooled for hemagglutinin (HA) and receptor-destroying enzyme (RDE) assays. Media of G clone cell cultures that developed characteristic cytopathic changes and positive

HA and RDE activities were pooled for stock preparations or subpassages. Media of BT or GBK cell cultures that developed any cytopathic changes were collected and subjected to 2nd or 3rd subpassages in the homologous cell culture systems to detect other respiratory bovine viruses. Media of uninoculated control cell cultures were similarly tested. The result of virus isolation was considered as negative when neither cytopathic changes nor HA and RDE activities were detected in the 3rd subpassage.

3.2.5 Identification of RBCV and Other Respiratory Bovine Virus Isolates

As reported (Storz et al., 1999), the virus isolates were initially identified by the characteristic cytopathic changes in the specifically permissive cell types. Additional differentiation tests involved: (1) assays for HA titers and RDE activities, a characteristic of RBCV, with rat red blood cells (RBC); (2) Cells infected with selected RBCV isolates that evidenced high fusogenic activity in the first passage were analyzed ultrastructurally. Fluids of such cultures also were examined by negative-contrast electron microscopy; (3) tests for HA activities of PI-3 with bovine and chicken RBC; (4) HA inhibition (HAI) tests with specific antisera for PI-3 and other virus isolates with bovine and chicken RBC; (5) virus infectivity neutralization tests with monospecific, polyclonal antiserum against BHV-1 which was diluted at 1:5; and (6) Immunofluorescence assay with fluorescent antibodies specific for BHV-1, PI-3, BVDV and BRSV.

3.2.6 Tests for HA Antigens and RDE

As reported (Storz et al., 1999; Storz et al., 2000b), washed rat, chicken or bovine RBC were prepared to 0.5% with PBS pH7.4, containing 0.05% bovine serum albumin (BSA). Fifty µl of each test samples was used and prepared as serial 2-fold dilutions in 96-well V-bottom microtiter plates (Costar, Cambridge, Mass.) with a volume of 50 µl. The prepared RBC suspensions were added in a volume of 50 µl. The plates were shaken to disperse the RBC suspensions and incubated at 6°C for 2 h. The HA titers were determined as the highest dilutions with complete aggregation of the RBC. The plates were then shifted to a 37°C incubator and placed there for 2 h. The RDE activities were considered as the highest dilutions with complete elution of adsorbed virions, that is the deaggregation of existing virus-erythrocyte complexes.

3.2.7 Hemagglutinin Inhibition Assay

As reported (Storz et al., 2000b), specific antisera for BCV (serum 1745) (Storz and Rott, 1980; Storz and Rott, 1981), PI-3 (serum 70P-1096) and other virus isolates were diluted at 1:4 in PBS, and then diluted in a serial 2-fold steps as 50 µl aliquots. Aliquots of media of cell cultures infected with suspected PI-3 or other virus isolates were collected, prepared to 16 HA units in PBS pH7.4 containing 0.05% BSA and used as antigens. The prepared antigens were added in a volume of 50 µl to each serum dilution. Following 30 min-reaction of serum and antigen mixtures at room temperature, 50 µl of

rat, chicken or bovine RBC suspensions were added as described above. The plates were kept at 6°C for 2 h, and the HAI titers were determined as the highest dilutions with complete inhibition of the aggregation of RBC.

3.2.8 Plaque Titration of RBCV in Lung Tissues

The plaque assay was performed according to recently published procedure (Lin et al., 1997b), using 6-well flat-bottom tissue culture plates (Becton-Dickinson, Lincoln Park, N.J.) with G clone confluent monolayers. Briefly, cells were maintained in DMEM supplemented with 5% FBS. Confluent monolayers were washed 2 times with serum-free Dulbecco's PBS (calcium and magnesium free) before inocula were added. Dilutions of test samples in 10-fold sequential steps were prepared in serum-free DMEM. Cell monolayers were inoculated with 0.5 ml of the appropriate dilutions and incubated for 1 h at 37°C. Following excessive inocula had been removed, the monolayers were overlayed with 0.5% agarose (Ultrapure DNA Grade Agarose, Bio-Rad Laboratories, Richmond, Calif.) in trypsin- and serum-free DMEM. After the overlay medium had solidified at room temperature, plates were incubated at 37°C in a 5% CO_2 incubator for 2 to 4 days until cytopathic changes were observed and plaques were formed. Cells were then stained with 0.05% neutral red (Sigma Chemical Co.) in serum-free Dulbecco's PBS (calcium and magnesium free), and fixed with 4% (v/v)

formalin (J.T. Baker Chemical Co, Phillipsburg, N.J.) in PBS for plaque quantitation. Uninoculated cell cultures were used as negative controls.

3.2.9 Virus Infectivity Neutralization Tests

The neutralization tests were performed in 24-well plates (Becton-Dickinson Labware) with confluent monolayers of GBK cells. Cultures of cells were maintained in MEM (Sigma Chemical Co.) supplemented with 10% FBS. The media of BT or GBK cell cultures with specific features of cytopathic changes were pooled for stock preparations and subjected to a sequential 10-fold dilution in serum-free MEM. A volume of 0.1 ml of the appropriate viral dilutions was mixed with 0.1 ml of monospecific, polyclonal antiserum 4897-IBR-NBF against BHV-1 which was diluted at 1:5 in serum-free MEM, and the mixture reacted at room temperature for 30 min. Cell monolayers were inoculated as described and overlayed with 2% methylcellulose (Sigma Chemical Co.) in MEM buffered with 25 mM Hepes (Sigma Chemical Co.) and supplemented with 2% FBS and penicillin (100 units/ml) - streptomycin (100 µg/ml). Plates were incubated at 37°C in a 5% CO_2 incubator for 2 to 4 days until cytopathic changes were observed and plaques were formed. The monolayers were then fixed with a prepared solution (76% ethanol, 5% formaline, 5% glacial acetic acid). After the overlayers were removed, the cells were stained with Gram Crystal Violet (DIFCO Laboratories, Detroit, Mich.) for plaque counting. Infectivity titration in the presence of MEM alone and uninoculated cell cultures with or without serum were used as controls.

3.2.10 Indirect Immunofluorescence Assay

Indirect immunofluorescence assay (IFA) was performed according to standard methods. Briefly, exfoliative cytological preparations on glass slides were air-dried, and fixed with methanol for 20 min at room temperature and incubated with respiratory bovine virus-specific antisera (diluted at 1:50 in PBS) at 37°C for 1 h, then washed with PBS and stained with fluorescein isothiocyanate (FITC)-labeled affinity purified goat anti-bovine IgG (diluted 1:50, Kirkegaard & Perry Laboratories Inc. Gaithersburg, Md.) at 37°C for 1 h. The cytologic preparations were then counter stained with Evans blue, and examined with a UV microscope (Olympus AH-2, Olympus Optical Co. LTD, Japan).

3.2.11 Virus Purification and Negative-contrast Electron Microscopy

The virus purification procedure was performed according to Zhang et al. (Zhang et al., 1994). Briefly, infected G clone cultures with approximately 90% of the cells showing cytopathic expression were subjected to 3 cycles of freezing and thawing, and sonicating 4 times for 15 s at a power setting of 4 (Branson Sonifier cell disruptor 200, Branson Ultrasonics Co, Danbury, Conn.) and centrifugation at 1,500 × g for 30 min. Supernatant fluids were collected, and precipitated overnight at 4°C with 10% (w/v) polyethylene glycol 8,000 and 0.5 M NaCl in TNE buffer (100 mM Tris, 10 mM NaCl, 1 mM EDTA, pH 7.4), and harvested by centrifugation at 1,500 × g. A drop of the

concentrated sample was stained with 2% phosphotunstic acid in PBS on a formvar-coated copper grid and examined with a ZEIS EM10 electron microscope at 60 to 80 kv.

3.3 Results

3.3.1 Clinical Observations

The 100 cattle in the 1993 experiment exhibited respiratory signs, including cough or mild-to-moderate dyspnea or nasal discharge on their arrival at the feedlot. Nine of them had all of these signs. Rectal temperatures ranged from 38.4°C to 40.8°C and 39.8°C to 40.9°C for cattle in Kansas feedlot and Arizona feedlot, respectively. Another 63 cattle from the 1994 LSU Livestock show, clinics and winter pastures of Southern and Northwest Louisiana also displayed clinical signs of acute respiratory distress, increased respiratory rates, mucupurulent nasal discharge and fever.

The 105 calves of the 1997 experiment appeared clinically normal on their arrival and during their stay at the OBB. Three days after arrival at the ARS feedlot following a transport over a distance of 1932 kilometer, 93 of them developed nasal and eye discharges and fever with rectal temperatures ranging from 40°C to 45°C. Ten calves died 1 to 5 days after arrival at the feedlot that were 7 to 11 days post-arrival at the OBB. Seventy-nine of the remaining 83 sick cattle were treated vigorously with

antibiotics. Twelve of the 105 calves did not exhibit adverse clinical signs of respiratory tract diseases, therefore were not treated.

During the 1998 investigation, 39 of the 120 cattle showed evident respiratory signs with rectal temperatures of above 40°C on their arrival at the OBB. A total of 107 calves developed adverse signs of respiratory tract diseases after arrival the ARS feedlot and therefore treated with antibiotics while 16 of them died 1 to 32 days later that were 7 to 38 days post-arrival at the OBB. The remaining 13 calves appeared clinically normal.

3.3.2 Shedding of Viruses in Nasal Secretions of Cattle and Properties of RBCV Isolates

The 1993 experiment: Of the 100 cattle involved in the 1993 experiment, 42 nasally shed viruses as detected through the characteristic cytopathic changes in the 3 different cell culture systems (Table 3.1). Of these 42 viral isolates, 38 were G clone cell-dependent, and were not detected in GBK or BT cell cultures. Of 50 cattle in the Kansas feedlot, 32 yielded this unique G clone cell-dependent viral agent, while of 50 cattle in the Arizona feedlot, 6 had viral isolate cultured from nasal swab samples that multiplied only in G clone cells.

Nasal swab samples from 4 cattle in the Arizona feedlot also contained a viral agent that induced cytopathic changes, mainly cell fusion, in BT cells. These viral isolates exhibited activities in agglutination of chicken and bovine RBC which were inhibited

Table 3.1: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle in Kansas Feedlot and Arizona Feedlot

State of Origin	ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
		CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
K Feedlot									
Oklahoma	OK-2793	+ F	>256	<2	-	-	-	-	-
	OK-2482	-	<2	<2	-	-	-	-	-
	OK-1986	-	<2	<2	-	-	-	-	-
	OK-3484	+ F	>256	<2	-	-	-	-	-
	OK-0543	+ F	>256	64 (2)	-	-	-	-	-
	OK-0105	+ F	>256	<2	-	-	-	-	-
	OK-0286	+ F (2)	>256 (2)	32 (2)	-	-	-	-	-
	OK-0481	+ F (2)	>256 (2)	8 (2)	-	-	-	-	-
	OK-3845	+ F	>256	<2	-	-	-	-	-
	OK-0099	+ F	>256	<2	-	-	-	-	-
	OK-1910	-	<2	<2	-	-	-	-	-
	OK-2077	-	<2	<2	-	-	-	-	-
	OK-0518	+ F	>256	<2	-	-	-	-	-
	OK-0357	+ F	>256	<2	-	-	-	-	-
	OK-0519	+ F	>256	<2	-	-	-	-	-
	OK-3683	-	<2	<2	-	-	-	-	-
	OK-0471	-	<2	<2	-	-	-	-	-
	OK-1595	-	<2	<2	-	-	-	-	-
	OK-2480	+ F	>256	<2	-	-	-	-	-
	OK-0251	+ F	>256	<2	-	-	-	-	-
	OK-2120	-	<2	<2	-	-	-	-	-

Table 3.1: contd.

Samples were provided by Dr. Stine from Immtech Biologics, Bucyrus, KS; K: Kansas; A: Arizona; BT: bovine turbinate cells; GBK: Georgia bovine kidney cells; CPE: the cytopathic expression; +: have CPE; -: no CPE; F; fusion; HC: honeycomb formation; Number in brackets indicates the number of subpassage in which (1) CPE was first detected and RBCV was isolated, otherwise, RBCV was isolated in the 1st G clone cell passage; (2) HA and RDE titers were recorded, otherwise, they were recorded with RBCV in the 1st G clone cell passage; HA: hemagglutinin; RDE: receptor-destroying enzyme; RBCV: respiratory bovine coronavirus; BHV-1: bovine herpes virus-1; BAV: bovine adenovirus; cpBVDV: cytopathogenic bovine viral diarrhea virus; PI-3: parainfluenza virus-3; BRSV: bovine respiratory syncytial virus.

State of Origin	ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
		CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
Oklahoma	OK-2081	+ F, HC (2)	>256 (2)	32 (2)	-	-	-	-	-
	OK-3258	+ F	>256	<2	-	-	-	-	-
	OK-3176	+ F	>256	<2	-	-	-	-	-
	OK-0725	+ F	>256	<2	-	-	-	-	-
	OK-3771	-	<2	<2	-	-	-	-	-
	OK-0687	-	<2	<2	-	-	-	-	-
	OK-0142	-	<2	<2	-	-	-	-	-
	OK-0358	+ F	>256	<2	-	-	-	-	-
	OK-3568	+ F	>256	<2	-	-	-	-	-
	OK-NT	-	<2	<2	-	-	-	-	-
	OK-0926	+ F	>256	<2	-	-	-	-	-
	OK-0514	+ F	>256	64 (3)	-	-	-	-	-
Texas	TX-956	-	<2	<2	-	-	-	-	-
	TX-671	+ F	>256	128 (2)	-	-	-	-	-
	TX-1335	+ F (2)	>256 (2)	64 (3)	-	-	-	-	-
	TX-1594		<2	<2	-	-	-	-	-
	TX-284	+ F	>256	64 (2)	-	-	-	-	-
	TX-911	+ F (2)	>256 (2)	4 (3)	-	-	-	-	-
	TX-1533	+ F	>256	32 (2)	-	-	-	-	-
	TX-187	-	<2	<2	-	-	-	-	-
	TX-1395	+ F	64	<2	-	-	-	-	-
	TX-2385	+ F	>256	64 (2)	-	-	-	-	-
	TX-1456	-	<2	<2	-	-	-	-	-
Arkansas	AR-1356	+ F	>256	64 (3)	-	-	-	-	-
	AR-?ET-65	-	<2	<2	-	-	-	-	-
	AR-NT1	+ F	>256	<2	-	-	-	-	-
	AR-NT2	+ F (2)	>256 (2)	64 (2)	-	-	-	-	-
	AR-NT3	+ F	>256	<2	-	-	-	-	-
	AR-NT4	-	<2	<2	-	-	-	-	-
A Feedlot									
California	AZ-000098	-	<2	<2	-	-	-	-	-
	AZ-19085	-	<2	<2	-	-	-	-	-
	AZ-19522	-	<2	<2	-	-	-	-	-
	AZ-19529	-	<2	<2	-	-	-	-	-
	AZ-19548	-	<2	<2	-	-	-	-	-
	AZ-19692	-	<2	<2	-	-	-	-	-
	AZ-19766	+ F	128	64 (2)	-	-	-	-	-

Table 3.1: contd.

State of Origin	ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
		CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
California	AZ-19906	-	<2	<2	-	-	-	-	-
	AZ-19937	-	<2	<2	-	-	-	-	-
	AZ-19963	-	<2	<2	-	-	-	-	-
	AZ-19972	-	<2	<2	-	-	-	-	-
	AZ-19990	-	<2	<2	-	-	-	-	-
	AZ-24366	-	<2	<2	-	-	-	-	-
	AZ-23509	-	<2	<2	-	-	-	-	-
	AZ-23559	-	<2	<2	-	-	-	-	-
	AZ-23584	-	<2	<2	-	-	-	-	-
	AZ-20898	-	<2	<2	-	-	-	-	-
	AZ-23481	-	<2	<2	-	+	-	-	-
	AZ-24316	-	<2	<2	-	-	-	-	-
	AZ-24444	-	<2	<2	-	+	-	-	-
	AZ-24362	+ F (2)	>256 (2)	32 (2)	-	-	-	-	-
	AZ-24646	-	<2	<2	-	-	-	-	-
	AZ-25357	-	<2	<2	-	-	-	-	-
	AZ-26525	-	<2	<2	-	-	-	-	-
	AZ-26787	-	<2	<2	-	-	-	-	-
	AZ-28256	-	<2	<2	-	-	-	-	-
	AZ-28410	+ F	16, >256 (2)	64 (2)	-	-	-	-	-
	AZ-24619	+ F	128	64 (2)	-	-	-	-	-
	AZ-23489	-	<2	<2	-	-	-	-	-
	AZ-19996	-	<2	<2	-	-	-	-	-
	AZ-000997	-	<2	<2	-	+	-	-	-
	AZ-24239	-	<2	<2	-	-	-	-	-
	AZ-24382	-	<2	<2	-	-	-	-	-
	AZ-24337	-	<2	<2	-	-	-	-	-
	AZ-19847	-	<2	<2	-	-	-	-	-
	AZ-26649	+ F	>256	64 (2)	-	-	-	-	-
	AZ-19884	-	<2	<2	-	-	-	-	-
	AZ-26337	+ F	>256	8 (2)	-	-	-	-	-
	AZ-28414	-	<2	<2	-	-	-	-	-
	AZ-23170	-	<2	<2	-	-	-	-	-
	AZ-20641	-	<2	<2	-	+	-	-	-
	AZ-20899	-	<2	<2	-	-	-	-	-
	AZ-22913	-	<2	<2	-	-	-	-	-
	AZ-26532	-	<2	<2	-	-	-	-	-

Table 3.1: contd.

State of Origin	ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
		CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
California	AZ-23586	-	<2	<2	-	-	-	-	-
	AZ-19958	-	<2	<2	-	-	-	-	-
	AZ-19948	-	<2	<2	-	-	-	-	-
	AZ-000996	-	<2	<2	-	-	-	-	-
	AZ-25120	-	<2	<2	-	-	-	-	-
	AZ-19902	-	<2	<2	-	-	-	-	-
	Total	38/100			0/100	4/100	0/100	0/100	0/100

Table 3.1.

by antiserum against PI-3. The BT cells infected with these viral isolates also adsorbed chicken and bovine RBC, and fluorescence-stained with antibodies specific for PI-3, but not with antibodies specific for BHV-1, BVDV or BRSV. All these findings reflected properties of PI-3.

Properties of RBCV isolates: The majority of these G clone cell-dependent viral isolates induced abundant and extensive polykaryons in the 1st passage in G clone cells without the enhancement of trypsin, and fused cells detached rapidly from the monolayers as soon as fusion was formed. Therefore, large granular clumps of cellular floats were created in the cell culture medium. Additional HA assays indicated that 16 to >256 HA units could be detected from the media of cell cultures infected with these viral agents, only with mouse and rat RBC. The hemagglutination activities could be inhibited by bovine antiserum 1745 specific to BCV. The associated characteristic RDE functions were measured at titers ranging from <2 to 128. Furthermore, negative-contrast electron microscopic analysis of infected G clone cells indicated the presence of round and enveloped virions that had the diameter of 70 to 80 nm, and contained electron-dense cores and club-shaped projections of 12 to 17 nm in length (Fig. 3.1). Therefore, these viruses were identified as respiratory bovine coronaviruses (RBCV).

Nasal swab samples from show cattle, clinical cases, and cattle on winter pastures: Of the 29 cattle in the 1994 Louisiana State University Livestock show, 4 nasally shed G clone cell-dependent RBCV isolates, while 2 had BHV-1 isolates that

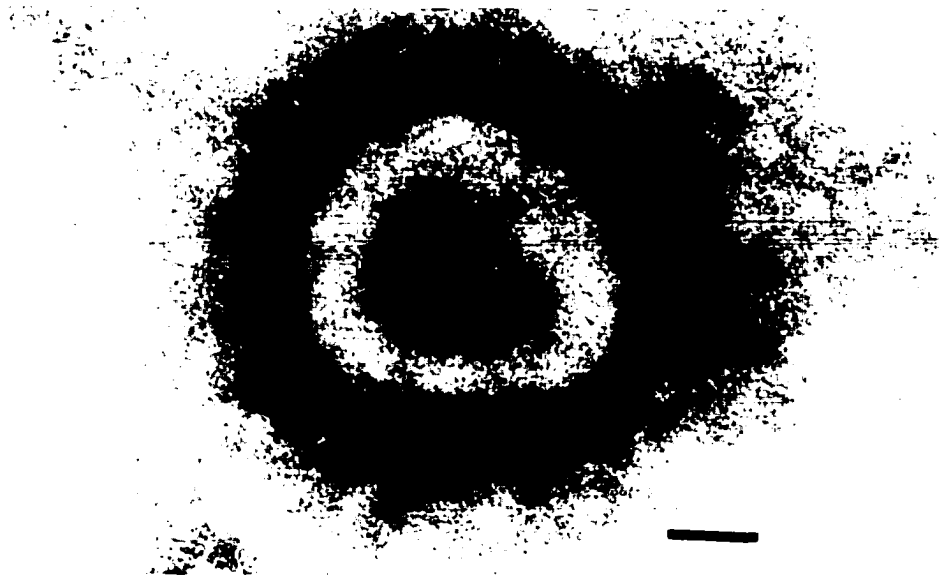


FIG. 3.1: Negatively stained viral particles of RBCV OK-0514-2.

Petal-shaped projections and short peplomeres are evident. Internal structures are not detectable (× 680,000); bar = 20μm.

multiplied in BT cells and formed pronounced grape-like aggregation of rounded cells (Table 3.2). These BHV-1 isolates were also identified by virus infectivity neutralization test and IFA with antisera specific to BHV-1. These calves had mixed infections with RBCV and BHV-1. One calf yielded bovine adenovirus in nasal swab samples through the characteristic cytopathic changes in BT cell cultures consisting of slow development of highly refractile swollen cells that gradually detached and lysed. This viral isolate is further identified by (1) virus infectivity neutralization test; (2) the presence of large, irregular intranuclear inclusion with varying halos while infected BT cells were stained with the Giemsa or hematoxylin-eosin; (3) lattice arrangement of circular viral capsid of 60 to 70 nm in diamer as indicated by ultrastructure analysis; and (4) absence of HA titers with rat and mouse RBC.

Of the 16 nasal swab samples provided by the clinicians of Louisiana State University and Mississippi State University, 2 contained G clone cell-dependent RBCV, while BHV-1 was detected once (Table 3.3).

Of the 15 cattle on the winter pasture of Southern Louisiana, 4 had RBCV isolates that multiplied only in the G clone cells, while 1 shed BHV-1 in nasal secretions (Table 3.4).

The 1997 shipping fever experiment: Of the 105 cattle, 64 nasally shed viruses dependent on G clone cells for replication on their arrival at the OBB, while BHV-1 was detected from 2 cattle which also had RBCV infection (Table 3.5). These G clone

Table 3.2: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle in the 1994 Louisiana State University Livestock Show

	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
ID of Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
LSU-94LSS-035-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-040-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-048-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-050-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-051-1	+ F	>256	32 (3)	-	-	-	-	-
LSU-94LSS-052-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-058-1	+ F	>256	32 (2)	-	-	-	-	-
LSU-94LSS-05B-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-063-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-062-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-081-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-082-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-094-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-092-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-091-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-095-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-099-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-112-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-114-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-119-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-128-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-141-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-142-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-143-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-144-1	+ F	>256	<2	+	-	-	-	-
LSU-94LSS-168-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-136-1	-	<2	<2	-	-	-	-	-
LSU-94LSS-193-1	+ F	>256	32 (2)	+	-	-	-	-
LSU-94LSS-218-1	-	<2	<2	-	-	-	+	-
Total	4/29			2/29	0/29	0/29	1/29	0/29

Notes as table 3.1.

Table 3.3: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle in Clinical Cases

ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
MS-VCS-249	-	<2	<2	-	-	-	-	-
MS-VCS-124	-	<2	<2	-	-	-	-	-
MS-VCS-18859	-	<2	<2	-	-	-	-	-
MS-VCS-18911	-	<2	<2	-	-	-	-	-
MS-VCS-33177	-	<2	<2	-	-	-	-	-
MS-VCS-37108	-	<2	<2	-	-	-	-	-
MS-VCS-125	+ F	>256	128 (2)	-	-	-	-	-
MS-VCS-3	-	<2	<2	-	-	-	-	-
MS-VCS-13	-	<2	<2	-	-	-	-	-
MS-VCS-20	-	<2	<2	+	-	-	-	-
MS-VCS-23	-	<2	<2	-	-	-	-	-
MS-VCS-123	-	<2	<2	-	-	-	-	-
LSU-VCS-6622698	+ F, HC	>256	32 (2)	-	-	-	-	-
LSU-VCS-625142	-	<2	<2	-	-	-	-	-
LSU-VMDL-625546	-	<2	<2	-	-	-	-	-
LSU-VMDL-96-2124	-	<2	<2	-	-	-	-	-
Total	2/16			1/16	0/16	0/16	0/16	0/16

MS-VCS: samples provided by Dr. Hoyt and Dr. Paulson from Department of Veterinary Clinical Sciences at Mississippi State University; LSU-VCS: samples provided by Dr. Angel from Department of Veterinary Clinical Sciences at Louisiana State University; LSU-VMDL: samples provided by Dr. Gill from Veterinary Medical Diagnostic Laboratory at Louisiana State University; Other notes as table 3.1.

Table 3.4: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle of the Lawton Ranch Herd

ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
96LR-1	+ F	32	<2	-	-	-	-	-
96LR-2	-	<2	<2	-	-	-	-	-
96LR-3	-	<2	<2	-	-	-	-	-
96LR-4	-	<2	<2	-	-	-	-	-
96LR-5	+ F	64, 256 (3)	<2 (3)	-	-	-	-	-
96LR-6	+ F	32, 128 (3)	32 (3)	-	-	-	-	-
96LR-7	+ F	32	32	-	-	-	-	-
96LR-8	-	<2	<2	-	-	-	-	-
96LR-9	-	<2	<2	-	-	-	-	-
96LR-10	-	<2	<2	-	-	-	-	-
96LR-11	-	<2	<2	-	-	-	-	-
96LR-12	-	<2	<2	-	-	-	-	-
96LR-13	-	<2	<2	-	-	-	-	-
96LR-14	-	<2	<2	-	-	-	-	-
96LR-15	-	<2	<2	+	-	-	-	-
Total	4/15			1/15	0/15	0/15	0/15	0/15

Samples were provided by Dr. Michael Meaux, Rhodes Veterinary Hospital, Inc., Sulphur, LA; LR: Lawton ranch; Other notes as table 3.1.

Table 3.5: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle at the Order-buyer Barn during the 1997 Shipping Fever Epizootic

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-1	-	<2	<2	-	-	-	-	-
97TXSF-2	-	<2	<2	-	-	-	-	-
97TXSF-3	-	<2	<2	-	-	-	-	-
97TXSF-4	-	<2	<2	-	-	-	-	-
97TXSF-5	+ F	32	4	-	-	-	-	-
97TXSF-6	+ F	64	4	-	-	-	-	-
97TXSF-7	-	<2	<2	-	-	-	-	-
97TXSF-8	+ F, HC	64	4	-	-	-	-	-
97TXSF-9	-	<2	<2	-	-	-	-	-
97TXSF-10	+ F, HC	128	4	-	-	-	-	-
97TXSF-11	+ F	64	4	-	-	-	-	-
97TXSF-12	+ F	64	4	-	-	-	-	-
97TXSF-13	+ F	64	4	-	-	-	-	-
97TXSF-14	+ F	64	4	-	-	-	-	-
97TXSF-15	+ F	64	4	-	-	-	-	-
97TXSF-16	-	<2	<2	-	-	-	-	-
97TXSF-17	-	<2	<2	-	-	-	-	-
97TXSF-18	+ F	64	<2	-	-	-	-	-
97TXSF-19	+ F, HC	32	2	-	-	-	-	-
97TXSF-20	+ F, HC	32	4	-	-	-	-	-
97TXSF-21	+ F	32	2	-	-	-	-	-
97TXSF-22	+ F, HC	32	2	-	-	-	-	-
97TXSF-23	-	<2	<2	-	-	-	-	-
97TXSF-24	+ F	32	<2	-	-	-	-	-
97TXSF-25	+ F	128	4	-	-	-	-	-
97TXSF-26	-	<2	<2	-	-	-	-	-
97TXSF-27	-	<2	<2	-	-	-	-	-
97TXSF-28	+ F	>256	>256	-	-	-	-	-
97TXSF-29	-	<2	<2	-	-	-	-	-

Table 3.5: contd.

Samples were supplied by Dr. Purdy at United States Department of Agriculture-Agriculture Research Service in Bushland, Texas. 97TXSF: nasal swab sample collected from cattle under investigation of shipping fever (SF) pneumonia at Texas (TX) in 1997 (97); Other notes as table 3.1.

ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-30	+ F	64	64	-	-	-	-	-
97TXSF-31	+ F, HC	128	64	-	-	-	-	-
97TXSF-32	-	<2	<2	-	-	-	-	-
97TXSF-33	+ F, HC	128	128	-	-	-	-	-
97TXSF-34	-	<2	<2	-	-	-	-	-
97TXSF-35	-	<2	<2	-	-	-	-	-
97TXSF-36	+ F, HC	128	128	-	-	-	-	-
97TXSF-37	+ F	>256	>256	-	-	-	-	-
97TXSF-38	+ F	>256	>256	-	-	-	-	-
97TXSF-39	+ F	64	64	-	-	-	-	-
97TXSF-40	+ F	64	64	-	-	-	-	-
97TXSF-41	-	<2	<2	-	-	-	-	-
97TXSF-42	+ F, HC	16	16	-	-	-	-	-
97TXSF-43	+ F, HC	32	32	-	-	-	-	-
97TXSF-44	+ F	16	16	-	-	-	-	-
97TXSF-45	+ F	32	32	-	-	-	-	-
97TXSF-46	+ F	16	<2	-	-	-	-	-
97TXSF-47	+ F, HC	64	64	-	-	-	-	-
97TXSF-48	+ F, HC	128	128	-	-	-	-	-
97TXSF-49	+ F	128	128	-	-	-	-	-
97TXSF-50	-	<2	<2	-	-	-	-	-
97TXSF-51	-	<2	<2	-	-	-	-	-
97TXSF-52	-	<2	<2	-	-	-	-	-
97TXSF-53	-	<2	<2	-	-	-	-	-
97TXSF-54	+ F, HC	64	64	-	-	-	-	-
97TXSF-55	-	<2	<2	-	-	-	-	-
97TXSF-56	-	<2	<2	-	-	-	-	-
97TXSF-57	-	<2	<2	-	-	-	-	-
97TXSF-58	+ F, HC	64	64	-	-	-	-	-
97TXSF-59	+ F	128	128	-	-	-	-	-
97TXSF-60	-	<2	<2	-	-	-	-	-
97TXSF-61	-	<2	<2	-	-	-	-	-
97TXSF-62	-	<2	<2	-	-	-	-	-
97TXSF-63	-	<2	<2	-	-	-	-	-
97TXSF-64	+ F	128	128	-	-	-	-	-
97TXSF-65	+ F, HC	64	64	-	-	-	-	-
97TXSF-66	-	<2	<2	-	-	-	-	-

Table 3.5: contd.

ID of	G Clone Cell Dependent-RBCV			BT or GBK Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-67	+ F, HC	32	32	-	-	-	-	-
97TXSF-68	-	<2	<2	-	-	-	-	-
97TXSF-69	-	<2	<2	-	-	-	-	-
97TXSF-70	+ F	64	64	+	-	-	-	-
97TXSF-71	+ F, HC	>256	>256	-	-	-	-	-
97TXSF-72	-	<2	<2	-	-	-	-	-
97TXSF-73	+ F, HC	>256	>256	+	-	-	-	-
97TXSF-74	+ F, HC	128	128	-	-	-	-	-
97TXSF-75	-	<2	<2	-	-	-	-	-
97TXSF-76	+ F	64	64	-	-	-	-	-
97TXSF-77	+ F, HC	64	64	-	-	-	-	-
97TXSF-78	-	<2	<2	-	-	-	-	-
97TXSF-79	+ F	128	128	-	-	-	-	-
97TXSF-80	+ F	128	128	-	-	-	-	-
97TXSF-81	+ F	32	32	-	-	-	-	-
97TXSF-82	-	<2	<2	-	-	-	-	-
97TXSF-83	-	<2	<2	-	-	-	-	-
97TXSF-84	+ F	64	64	-	-	-	-	-
97TXSF-85	+ F	128	128	-	-	-	-	-
97TXSF-86	-	<2	<2	-	-	-	-	-
97TXSF-87	+ F	32	32	-	-	-	-	-
97TXSF-88	-	<2	<2	-	-	-	-	-
97TXSF-89	+ F, HC	64	64	-	-	-	-	-
97TXSF-90	+ F	32	32	-	-	-	-	-
97TXSF-91	-	<2	<2	-	-	-	-	-
97TXSF-92	+ F	64	64	-	-	-	-	-
97TXSF-93	+ F	8	8	-	-	-	-	-
97TXSF-94	+ F	64	64	-	-	-	-	-
97TXSF-95	+ F	>256	>256	-	-	-	-	-
97TXSF-96	+ F	128	128	-	-	-	-	-
97TXSF-97	+ F	>256	>256	-	-	-	-	-
97TXSF-98	-	<2	<2	-	-	-	-	-
97TXSF-99	+ F	64	64	-	-	-	-	-
97TXSF-100	+ F	32	32	-	-	-	-	-
97TXSF-101	+ F	32	32	-	-	-	-	-
97TXSF-102	+ F	32	32	-	-	-	-	-
97TXSF-103	+ F	128	128	-	-	-	-	-

Table 3.5: contd.

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-104	-	<2	<2	-	-	-	-	-
97TXSF-105	-	<2	<2	-	-	-	-	-
Total	64/105			2/105	0/105	0/105	0/105	0/105

Table 3.5.

cell-dependent RBCV strains were isolated in the 1st attempt after inoculating the cells with clinical specimens. During transport to the ARS feed yard, 14 discontinued shedding RBCV while an additional 22 cattle contracted RBCV infection during transport to the ARS feed yard (Table 3.6). Therefore, 72 cattle contained RBCV in nasal secretions right after their arrived at the feed yard, which was 7 days post-arrival at the OBB, when the calves exhibited severe clinical signs of respiratory tract diseases. Meanwhile, no other respiratory bovine viruses were detected in the cattle. A total of 86 cattle experienced RBCV infection during the first week of this epizootic. Of the remaining 95 surviving cattle, the numbers of cattle nasally shedding RBCV dramatically reduced to 5 and 4 on days 14 and 21 post-arrival at the OBB, respectively, when adverse clinical signs were not evident (Tables 3.7 and 3.8). The single BHV-1 infections were detected from nasal swab samples of 4 cattle on day 14 post-arrival at the OBB, while 1 and 4 cattle shed BHV-1 only and PI-3 only, respectively, on day 21 post-arrival at the OBB. The BRSV, cytopathogenic BVDV and bovine adenovirus could not be detected from all nasal swab samples collected during the 1997 epizootic.

The 1998 shipping fever experiment: Of the 120 cattle, 89 contained RBCV isolates in nasal secretions on their arrival at the OBB, when adverse clinical signs were already pronounced, and aggressive antibiotic treatment was applied (Table 3.9). Only 1 cattle had BHV-1 infection at this time. During transport to the ARS feed yard, 2 calves became infected with RBCV while 24 calves stopped shedding this virus (Table 3.10).

Table 3.6: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 7 Post-arrival at the Order-buyer Barn during the 1997 Shipping Fever Epizootic

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-1	-	<2	<2	-	-	-	-	-
97TXSF-2	-	<2	<2	-	-	-	-	-
97TXSF-3	+ F, HC	16	<2	-	-	-	-	-
97TXSF-4	-	<2	<2	-	-	-	-	-
97TXSF-5	+ F, HC	64	<2	-	-	-	-	-
97TXSF-6	+ F, HC	64	<2	-	-	-	-	-
97TXSF-7	-	<2	<2	-	-	-	-	-
97TXSF-8	+ F, HC	128	8	-	-	-	-	-
97TXSF-9	-	<2	<2	-	-	-	-	-
97TXSF-10	+ F, HC	16	16	-	-	-	-	-
97TXSF-11	+ F, HC	128	128	-	-	-	-	-
97TXSF-12	-	<2	<2	-	-	-	-	-
97TXSF-13	+ F	64	2	-	-	-	-	-
97TXSF-14	+ F, HC	64	<2	-	-	-	-	-
97TXSF-15	+ F	64	<2	-	-	-	-	-
97TXSF-16	-	<2	<2	-	-	-	-	-
97TXSF-17	+ F	32	32	-	-	-	-	-
97TXSF-18	-	<2	<2	-	-	-	-	-
97TXSF-19	+ F	128	<2	-	-	-	-	-
97TXSF-20	+ F	64	<2	-	-	-	-	-
97TXSF-21	+ F	64	<2	-	-	-	-	-
97TXSF-22	-	<2	<2	-	-	-	-	-
97TXSF-23	-	<2	<2	-	-	-	-	-
97TXSF-24	-	<2	<2	-	-	-	-	-
97TXSF-25	+ F	64	<2	-	-	-	-	-
97TXSF-26	+ F, HC	64	64	-	-	-	-	-
97TXSF-27	-	<2	<2	-	-	-	-	-
97TXSF-28	+ F, HC	64	4	-	-	-	-	-
97TXSF-29	-	<2	<2	-	-	-	-	-
97TXSF-30	-	<2	<2	-	-	-	-	-
97TXSF-31	+ F, HC	64	16	-	-	-	-	-

Table 3.6: contd.

Notes as table 3.5.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-32	-	<2	<2	-	-	-	-	-
97TXSF-33	-	<2	<2	-	-	-	-	-
97TXSF-34	-	<2	<2	-	-	-	-	-
97TXSF-35	-	<2	<2	-	-	-	-	-
97TXSF-36	+ F, HC	128	128	-	-	-	-	-
97TXSF-37	-	<2	<2	-	-	-	-	-
97TXSF-38	+ F, HC	64	2	-	-	-	-	-
97TXSF-39	-	<2	<2	-	-	-	-	-
97TXSF-40	+ F	16	2	-	-	-	-	-
97TXSF-41	-	<2	<2	-	-	-	-	-
97TXSF-42	+ F	32	4	-	-	-	-	-
97TXSF-43	+ F	32	<2	-	-	-	-	-
97TXSF-44	+ F	64	64	-	-	-	-	-
97TXSF-45	-	<2	<2	-	-	-	-	-
97TXSF-46	-	<2	<2	-	-	-	-	-
97TXSF-47	+ F, HC	64	<2	-	-	-	-	-
97TXSF-48	-	<2	<2	-	-	-	-	-
97TXSF-49	-	<2	<2	-	-	-	-	-
97TXSF-50	+ F	64	8	-	-	-	-	-
97TXSF-51	+ F	64	4	-	-	-	-	-
97TXSF-52	+ F, HC	32	<2	-	-	-	-	-
97TXSF-53	+ F, HC	32	4	-	-	-	-	-
97TXSF-54	+ F	64	64	-	-	-	-	-
97TXSF-55	-	<2	<2	-	-	-	-	-
97TXSF-56	+ F, HC	64	<2	-	-	-	-	-
97TXSF-57	+ F	64	64	-	-	-	-	-
97TXSF-58	+ F, HC	128	<2	-	-	-	-	-
97TXSF-59	+ F, HC	64	<2	-	-	-	-	-
97TXSF-60	+ F, HC	64	<2	-	-	-	-	-
97TXSF-61	+ F, HC	16	16	-	-	-	-	-
97TXSF-62	+ F, HC	128	128	-	-	-	-	-
97TXSF-63	-	<2	<2	-	-	-	-	-
97TXSF-64	+ F, HC	128	<2	-	-	-	-	-
97TXSF-65	+ F	64	64	-	-	-	-	-
97TXSF-66	+ F, HC	128	8	-	-	-	-	-
97TXSF-67	+ F	32	<2	-	-	-	-	-
97TXSF-68	+ F	32	32	-	-	-	-	-

Table 3.6: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-69	-	<2	<2	-	-	-	-	-
97TXSF-70	+ F	32	<2	-	-	-	-	-
97TXSF-71	+ F	64	<2	-	-	-	-	-
97TXSF-72	-	<2	<2	-	-	-	-	-
97TXSF-73	+ F, HC	64	<2	-	-	-	-	-
97TXSF-74	-	<2	<2	-	-	-	-	-
97TXSF-75	+ F	32	<2	-	-	-	-	-
97TXSF-76	+ F, HC	64	64	-	-	-	-	-
97TXSF-77	+ F, HC	128	2	-	-	-	-	-
97TXSF-78	+ F	128	<2	-	-	-	-	-
97TXSF-79	+ F	32	32	-	-	-	-	-
97TXSF-80	+ F	64	64	-	-	-	-	-
97TXSF-81	+ F	64	2	-	-	-	-	-
97TXSF-82	+ F	64	64	-	-	-	-	-
97TXSF-83	+ F	64	64	-	-	-	-	-
97TXSF-84	+ F	32	<2	-	-	-	-	-
97TXSF-85	+ F	32	<2	-	-	-	-	-
97TXSF-86	-	<2	<2	-	-	-	-	-
97TXSF-87	+ F	128	2	-	-	-	-	-
97TXSF-88	-	<2	<2	-	-	-	-	-
97TXSF-89	+ F	128	8	-	-	-	-	-
97TXSF-90	-	<2	<2	-	-	-	-	-
97TXSF-91	+ F	64	64	-	-	-	-	-
97TXSF-92	+ F	64	4	-	-	-	-	-
97TXSF-93	+ F, HC	64	64	-	-	-	-	-
97TXSF-94	+ F	64	4	-	-	-	-	-
97TXSF-95	+ F, HC	32	<2	-	-	-	-	-
97TXSF-96	+ F	16	2	-	-	-	-	-
97TXSF-97	+ F, HC	64	2	-	-	-	-	-
97TXSF-98	+ F, HC	64	64	-	-	-	-	-
97TXSF-99	+ F	64	2	-	-	-	-	-
97TXSF-100	+ F, HC	64	8	-	-	-	-	-
97TXSF-101	+ F	64	4	-	-	-	-	-
97TXSF-102	+ F, HC	32	4	-	-	-	-	-
97TXSF-103	+ F, HC	64	4	-	-	-	-	-
97TXSF-104	+ F, HC	32	4	-	-	-	-	-
97TXSF-105	+ F	128	4	-	-	-	-	-
Total	72/105			0/105	0/105	0/105	0/105	0/105

Table 3.6.

Table 3.7: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 14 Post-arrival at the Order-buyer Barn during the 1997 Shipping Fever Epizootic

ID of Cattle	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-1	-	<2	<2	-	-	-	-	-
97TXSF-2	-	<2	<2	-	-	-	-	-
97TXSF-3	-	<2	<2	-	-	-	-	-
97TXSF-4	-	<2	<2	+	-	-	-	-
97TXSF-5	Died							
97TXSF-6	+ F (2)	128	<2	-	-	-	-	-
97TXSF-7	-	<2	<2	-	-	-	-	-
97TXSF-8	-	<2	<2	-	-	-	-	-
97TXSF-9	-	<2	<2	+	-	-	-	-
97TXSF-10	-	<2	<2	-	-	-	-	-
97TXSF-11	-	<2	<2	-	-	-	-	-
97TXSF-12	-	<2	<2	-	-	-	-	-
97TXSF-13	-	<2	<2	-	-	-	-	-
97TXSF-14	-	<2	<2	-	-	-	-	-
97TXSF-15	Died							
97TXSF-16	-	<2	<2	-	-	-	-	-
97TXSF-17	-	<2	<2	+	-	-	-	-
97TXSF-18	-	<2	<2	-	-	-	-	-
97TXSF-19	Died							
97TXSF-20	-	<2	<2	-	-	-	-	-
97TXSF-21	-	<2	<2	-	-	-	-	-
97TXSF-22	-	<2	<2	-	-	-	-	-
97TXSF-23	-	<2	<2	-	-	-	-	-
97TXSF-24	-	<2	<2	-	-	-	-	-
97TXSF-25	-	<2	<2	-	-	-	-	-
97TXSF-26	-	<2	<2	-	-	-	-	-
97TXSF-27	-	<2	<2	-	-	-	-	-
97TXSF-28	-	<2	<2	-	-	-	-	-
97TXSF-29	-	<2	<2	-	-	-	-	-
97TXSF-30	-	<2	<2	-	-	-	-	-
97TXSF-31	-	<2	<2	-	-	-	-	-
97TXSF-32	-	<2	<2	-	-	-	-	-

Table 3.7: contd.

Notes as table 3.5.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-33	+ F (2)	32, >256 (2)	<2	-	-	-	-	-
97TXSF-34	-	<2	<2	-	-	-	-	-
97TXSF-35	-	<2	<2	-	-	-	-	-
97TXSF-36	+ F, HC	16	4	-	-	-	-	-
97TXSF-37	-	<2	<2	-	-	-	-	-
97TXSF-38	-	<2	<2	-	-	-	-	-
97TXSF-39	-	<2	<2	-	-	-	-	-
97TXSF-40	-	<2	<2	-	-	-	-	-
97TXSF-41	-	<2	<2	-	-	-	-	-
97TXSF-42	-	<2	<2	-	-	-	-	-
97TXSF-43	Died							
97TXSF-44	-	<2	<2	-	-	-	-	-
97TXSF-45	+ F	64	4	-	-	-	-	-
97TXSF-46	-	<2	<2	-	-	-	-	-
97TXSF-47	-	<2	<2	-	-	-	-	-
97TXSF-48	-	<2	<2	-	-	-	-	-
97TXSF-49	-	<2	<2	-	-	-	-	-
97TXSF-50	-	<2	<2	-	-	-	-	-
97TXSF-51	-	<2	<2	-	-	-	-	-
97TXSF-52	Died							
97TXSF-53	+ F	>256	>256	-	-	-	-	-
97TXSF-54	-	<2	<2	-	-	-	-	-
97TXSF-55	-	<2	<2	-	-	-	-	-
97TXSF-56	-	<2	<2	-	-	-	-	-
97TXSF-57	-	<2	<2	-	-	-	-	-
97TXSF-58	Died							
97TXSF-59	-	<2	<2	-	-	-	-	-
97TXSF-60	-	<2	<2	-	-	-	-	-
97TXSF-61	-	<2	<2	-	-	-	-	-
97TXSF-62	Died							
97TXSF-63	Died							
97TXSF-64	-	<2	<2	-	-	-	-	-
97TXSF-65	-	<2	<2	+	-	-	-	-
97TXSF-66	-	<2	<2	-	-	-	-	-
97TXSF-67	-	<2	<2	-	-	-	-	-
97TXSF-68	-	<2	<2	-	-	-	-	-
97TXSF-69	-	<2	<2	-	-	-	-	-

Table 3.7: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-70	-	<2	<2	-	-	-	-	-
97TXSF-71	-	<2	<2	-	-	-	-	-
97TXSF-72	-	<2	<2	-	-	-	-	-
97TXSF-73	-	<2	<2	-	-	-	-	-
97TXSF-74	-	<2	<2	-	-	-	-	-
97TXSF-75	Died							
97TXSF-76	-	<2	<2	-	-	-	-	-
97TXSF-77	-	<2	<2	-	-	-	-	-
97TXSF-78	-	<2	<2	-	-	-	-	-
97TXSF-79	-	<2	<2	-	-	-	-	-
97TXSF-80	-	<2	<2	-	-	-	-	-
97TXSF-81	-	<2	<2	-	-	-	-	-
97TXSF-82	-	<2	<2	-	-	-	-	-
97TXSF-83	-	<2	<2	-	-	-	-	-
97TXSF-84	-	<2	<2	-	-	-	-	-
97TXSF-85	-	<2	<2	-	-	-	-	-
97TXSF-86	-	<2	<2	-	-	-	-	-
97TXSF-87	-	<2	<2	-	-	-	-	-
97TXSF-88	-	<2	<2	-	-	-	-	-
97TXSF-89	-	<2	<2	-	-	-	-	-
97TXSF-90	-	<2	<2	-	-	-	-	-
97TXSF-91	-	<2	<2	-	-	-	-	-
97TXSF-92	-	<2	<2	-	-	-	-	-
97TXSF-93	-	<2	<2	-	-	-	-	-
97TXSF-94	-	<2	<2	-	-	-	-	-
97TXSF-95	-	<2	<2	-	-	-	-	-
97TXSF-96	Died							
97TXSF-97	-	<2	<2	-	-	-	-	-
97TXSF-98	-	<2	<2	-	-	-	-	-
97TXSF-99	-	<2	<2	-	-	-	-	-
97TXSF-100	-	<2	<2	-	-	-	-	-
97TXSF-101	-	<2	<2	-	-	-	-	-
97TXSF-102	-	<2	<2	-	-	-	-	-
97TXSF-103	-	<2	<2	-	-	-	-	-
97TXSF-104	-	<2	<2	-	-	-	-	-
97TXSF-105	-	<2	<2	-	-	-	-	-
Total	5/95			4/95	0/95	0/95	0/95	0/95

Table 3.7.

Table 3.8: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 21 Post-arrival at the Order-buyer Barn during the 1997 Shipping Fever Epizootic

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-1	-	<2	<2	-	-	-	-	-
97TXSF-2	-	<2	<2	-	-	-	-	-
97TXSF-3	-	<2	<2	-	-	-	-	-
97TXSF-4	-	<2	<2	-	-	-	-	-
97TXSF-5	Died							
97TXSF-6	-	<2	<2	-	-	-	-	-
97TXSF-7	-	<2	<2	-	-	-	-	-
97TXSF-8	-	<2	<2	-	-	-	-	-
97TXSF-9	-	<2	<2		-	-	-	-
97TXSF-10	-	<2	<2	-	-	-	-	-
97TXSF-11	-	<2	<2	-	-	-	-	-
97TXSF-12	-	<2	<2	-	-	-	-	-
97TXSF-13	-	<2	<2	-	-	-	-	-
97TXSF-14	-	<2	<2	-	-	-	-	-
97TXSF-15	Died							
97TXSF-16	-	<2	<2	-	-	-	-	-
97TXSF-17	-	<2	<2	-	-	-	-	-
97TXSF-18	-	<2	<2	-	-	-	-	-
97TXSF-19	Died							
97TXSF-20	-	<2	<2	-	-	-	-	-
97TXSF-21	-	<2	<2	-	-	-	-	-
97TXSF-22	-	<2	<2	-	+	-	-	-
97TXSF-23	-	<2	<2	-	-	-	-	-
97TXSF-24	-	<2	<2	-	-	-	-	-
97TXSF-25	-	<2	<2	-	-	-	-	-
97TXSF-26	-	<2	<2	-	-	-	-	-
97TXSF-27	-	<2	<2	-	-	-	-	-
97TXSF-28	+ F, HC	256	<2	-	-	-	-	-
97TXSF-29	-	<2	<2	-	+	-	-	-
97TXSF-30	-	<2	<2	-	-	-	-	-
97TXSF-31	-	<2	<2	-	-	-	-	-
97TXSF-32	-	<2	<2	-	-	-	-	-

Table 3.8: contd.

Notes as table 3.5.

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-33	-	<2	<2	-	-	-	-	-
97TXSF-34	-	<2	<2	-	-	-	-	-
97TXSF-35	-	<2	<2	-	-	-	-	-
97TXSF-36	-	<2	<2	-	-	-	-	-
97TXSF-37	-	<2	<2	-	-	-	-	-
97TXSF-38	-	<2	<2	-	-	-	-	-
97TXSF-39	-	<2	<2	-	-	-	-	-
97TXSF-40	-	<2	<2	-	-	-	-	-
97TXSF-41	-	<2	<2	-	+	-	-	-
97TXSF-42	-	<2	<2	-	-	-	-	-
97TXSF-43	Died							
97TXSF-44	-	<2	<2	-	-	-	-	-
97TXSF-45	-	<2	<2	-	-	-	-	-
97TXSF-46	-	<2	<2	-	-	-	-	-
97TXSF-47	-	<2	<2	-	-	-	-	-
97TXSF-48	-	<2	<2	-	-	-	-	-
97TXSF-49	-	<2	<2	-	-	-	-	-
97TXSF-50	-	<2	<2	-	-	-	-	-
97TXSF-51	-	<2	<2	-	-	-	-	-
97TXSF-52	Died							
97TXSF-53	-	<2	<2	-	-	-	-	-
97TXSF-54	-	<2	<2	+	-	-	-	-
97TXSF-55	-	<2	<2	-	-	-	-	-
97TXSF-56	-	<2	<2	-	-	-	-	-
97TXSF-57	-	<2	<2	-	-	-	-	-
97TXSF-58	Died							
97TXSF-59	-	<2	<2	-	-	-	-	-
97TXSF-60	-	<2	<2	-	-	-	-	-
97TXSF-61	-	<2	<2	-	-	-	-	-
97TXSF-62	Died							
97TXSF-63	Died							
97TXSF-64	-	<2	<2	-	-	-	-	-
97TXSF-65	+ F, HC	4	<2	-	-	-	-	-
97TXSF-66	-	<2	<2	-	-	-	-	-
97TXSF-67	-	<2	<2	-	-	-	-	-
97TXSF-68	-	<2	<2	-	-	-	-	-
97TXSF-69	-	<2	<2	-	-	-	-	-

Table 3.8: contd.

ID of	G Clone Cell Dependent-RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
97TXSF-70	-	<2	<2	-	-	-	-	-
97TXSF-71	-	<2	<2	-	-	-	-	-
97TXSF-72	-	<2	<2	-	-	-	-	-
97TXSF-73	-	<2	<2	-	-	-	-	-
97TXSF-74	-	<2	<2	-	-	-	-	-
97TXSF-75	Died							
97TXSF-76	-	<2	<2	-	-	-	-	-
97TXSF-77	-	<2	<2	-	-	-	-	-
97TXSF-78	-	<2	<2	-	-	-	-	-
97TXSF-79	-	<2	<2	-	-	-	-	-
97TXSF-80	-	<2	<2	-	-	-	-	-
97TXSF-81	+ F	64	<2	-	-	-	-	-
97TXSF-82	+ F, HC	64	4	-	-	-	-	-
97TXSF-83	-	<2	<2	-	-	-	-	-
97TXSF-84	-	<2	<2	-	-	-	-	-
97TXSF-85	-	<2	<2	-	+	-	-	-
97TXSF-86	-	<2	<2	-	-	-	-	-
97TXSF-87	-	<2	<2	-	-	-	-	-
97TXSF-88	-	<2	<2	-	-	-	-	-
97TXSF-89	-	<2	<2	-	-	-	-	-
97TXSF-90	-	<2	<2	-	-	-	-	-
97TXSF-91	-	<2	<2	-	-	-	-	-
97TXSF-92	-	<2	<2	-	-	-	-	-
97TXSF-93	-	<2	<2	-	-	-	-	-
97TXSF-94	-	<2	<2	-	-	-	-	-
97TXSF-95	-	<2	<2	-	-	-	-	-
97TXSF-96	Died							
97TXSF-97	-	<2	<2	-	-	-	-	-
97TXSF-98	-	<2	<2	-	-	-	-	-
97TXSF-99	-	<2	<2	-	-	-	-	-
97TXSF-100	-	<2	<2	-	-	-	-	-
97TXSF-101	-	<2	<2	-	-	-	-	-
97TXSF-102	-	<2	<2	-	-	-	-	-
97TXSF-103	-	<2	<2	-	-	-	-	-
97TXSF-104	-	<2	<2	-	-	-	-	-
97TXSF-105	-	<2	<2	-	-	-	-	-
Total	4/95			1/95	4/95	0/95	0/95	0/95

Table 3.8.

Table 3.9: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle at the Order-buyer Barn during the 1998 Shipping Fever Epizootic

ID of	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-1	+ F	>256	<2	-	-	-	-	-
98TXSF-2	-	<2	<2	-	-	-	-	-
98TXSF-3	-	<2	<2	-	-	-	-	-
98TXSF-4	+ F	>256	>256	-	-	-	-	-
98TXSF-5	+ F	>256	>256	-	-	-	-	-
98TXSF-6	+ F	>256	2	-	-	-	-	-
98TXSF-7	+ F	>256	>256	-	-	-	-	-
98TXSF-8	+ F	>256	<2	-	-	-	-	-
98TXSF-9	+ F	>256	<2	-	-	-	-	-
98TXSF-10	+ F	>256	128	-	-	-	-	-
98TXSF-11	+ F	>256	64	-	-	-	-	-
98TXSF-12	+ F	>256	2	-	-	-	-	-
98TXSF-13	+ F	>256	64	-	-	-	-	-
98TXSF-14	+ F	128	128	-	-	-	-	-
98TXSF-15	+ F	>256	128	-	-	-	-	-
98TXSF-16	+ F	>256	8	-	-	-	-	-
98TXSF-17	+ F	>256	<2	-	-	-	-	-
98TXSF-18	+ F	>256	<2	-	-	-	-	-
98TXSF-19	+ F	>256	32	-	-	-	-	-
98TXSF-20	+ F	>256	<2	-	-	-	-	-
98TXSF-21	+ F	>256	<2	-	-	-	-	-
98TXSF-22	-	<2	<2	-	-	-	-	-
98TXSF-23	+ F	>256	<2	-	-	-	-	-
98TXSF-24	+ F	>256	<2	-	-	-	-	-
98TXSF-25	+ F	>256	>256	-	-	-	-	-
98TXSF-26	+ F	>256	64	-	-	-	-	-
98TXSF-27	-	<2	<2	-	-	-	-	-
98TXSF-28	+ F	>256	>256	-	-	-	-	-
98TXSF-29	+ F	>256	32	-	-	-	-	-

Table 3.9: contd.

Samples were supplied by Dr. Purdy at United States Department of Agriculture-Agriculture Research Service in Bushland, Texas. 98TXSF: nasal swab sample collected from cattle under investigation of shipping fever (SF) pneumonia at Texas (TX) in 1998 (98); N/A: not available; Other notes as table 3.1.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-30	-	<2	<2	-	-	-	-	-
98TXSF-31	+ F	>256	<2	-	-	-	-	-
98TXSF-32	-	<2	<2	-	-	-	-	-
98TXSF-33	+ F	>256	<2	-	-	-	-	-
98TXSF-34	-	<2	<2	-	-	-	-	-
98TXSF-35	+ F	>256	<2	-	-	-	-	-
98TXSF-36	+ F	>256	32	-	-	-	-	-
98TXSF-37	+ F	>256	<2	-	-	-	-	-
98TXSF-38	-	<2	<2	-	-	-	-	-
98TXSF-39	+ F	>256	>256	-	-	-	-	-
98TXSF-40	+ F	>256	<2	-	-	-	-	-
98TXSF-41	-	<2	<2	-	-	-	-	-
98TXSF-42	+ F	>256	<2	-	-	-	-	-
98TXSF-43	+ F	>256	<2	-	-	-	-	-
98TXSF-44	-	<2	<2	-	-	-	-	-
98TXSF-45	+ F	>256	<2	-	-	-	-	-
98TXSF-46	+ F	>256	<2	-	-	-	-	-
98TXSF-47	-	<2	<2	-	-	-	-	-
98TXSF-48	+ F	>256	>256	-	-	-	-	-
98TXSF-49	+ F	>256	<2	-	-	-	-	-
98TXSF-50	+ F	>256	<2	-	-	-	-	-
98TXSF-51	+ F	>256	<2	-	-	-	-	-
98TXSF-52	-	<2	<2	-	-	-	-	-
98TXSF-53	+ F	>256	<2	-	-	-	-	-
98TXSF-54	+ F	>256	<2	-	-	-	-	-
98TXSF-55	+ F	>256	>256	-	-	-	-	-
98TXSF-56	+ F	>256	<2	-	-	-	-	-
98TXSF-57	+ F	>256	<2	-	-	-	-	-
98TXSF-58	-	<2	<2	-	-	-	-	-
98TXSF-59	+ F	>256	<2	-	-	-	-	-
98TXSF-60	+ F	>256	<2	-	-	-	-	-
98TXSF-61	+F	>256	<2	-	-	-	-	-
98TXSF-62	-	<2	<2	-	-	-	-	-
98TXSF-63	+ F	>256	<2	-	-	-	-	-
98TXSF-64	N/A							
98TXSF-65	+ F	>256	>256	-	-	-	-	-
98TXSF-66	+ F	>256	>256	-	-	-	-	-

Table 3.9: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-67	+ F	>256	>256	-	-	-	-	-
98TXSF-68	+ F	>256	>256	-	-	-	-	-
98TXSF-69	+ F	>256	>256	-	-	-	-	-
98TXSF-70	-	<2	<2	-	-	-	-	-
98TXSF-71	+ F	>256	>256	-	-	-	-	-
98TXSF-72	+ F	>256	>256	-	-	-	-	-
98TXSF-73	+ F	>256	>256	-	-	-	-	-
98TXSF-74	-	<2	<2	-	-	-	-	-
98TXSF-75	+ F	>256	>256	-	-	-	-	-
98TXSF-76	+ F	>256	>256	-	-	-	-	-
98TXSF-77	+ F	>256	>256	-	-	-	-	-
98TXSF-78	-	<2	<2	-	-	-	-	-
98TXSF-79	+ F	>256	>256	-	-	-	-	-
98TXSF-80	+ F	>256	>256	-	-	-	-	-
98TXSF-81	+ F	>256	16	-	-	-	-	-
98TXSF-82	+ F	>256	>256	-	-	-	-	-
98TXSF-83	+ F	>256	>256	-	-	-	-	-
98TXSF-84	-	<2	<2	-	-	-	-	-
98TXSF-85	-	<2	<2	-	-	-	-	-
98TXSF-86	-	<2	<2	-	-	-	-	-
98TXSF-87	-	<2	<2	-	-	-	-	-
98TXSF-88	-	<2	<2	-	-	-	-	-
98TXSF-89	+ F, HC	>256	>256	-	-	-	-	-
98TXSF-90	-	<2	<2	-	-	-	-	-
98TXSF-91	+ F	>256	>256	-	-	-	-	-
98TXSF-92	+ F	>256	>256	-	-	-	-	-
98TXSF-93	-	<2	<2	-	-	-	-	-
98TXSF-94	-	<2	<2	+	-	-	-	-
98TXSF-95	-	<2	<2	-	-	-	-	-
98TXSF-96	+ F, HC	>256	>256	-	-	-	-	-
98TXSF-97	+ F	> 256	>256	-	-	-	-	-
98TXSF-98	-	<2	<2	-	-	-	-	-
98TXSF-99	+ F	256	256	-	-	-	-	-
98TXSF-100	+ F	>256	>256	-	-	-	-	-
98TXSF-101	+ F	>256	>256	-	-	-	-	-
98TXSF-102	+ F	>256	>256	-	-	-	-	-
98TXSF-103	+ F	>256	>256	-	-	-	-	-
98TXSF-104	+ F	256	256	-	-	-	-	-

Table 3.9: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-105	+ F	>256	>256	-	-	-	-	-
98TXSF-106	+ F	>256	>256	-	-	-	-	-
98TXSF-107	+ F	>256	>256	-	-	-	-	-
98TXSF-108	+ F	>256	>256	-	-	-	-	-
98TXSF-109	+ F	>256	>256	-	-	-	-	-
98TXSF-110	+ F	>256	>256	-	-	-	-	-
98TXSF-111	+ F	256	256	-	-	-	-	-
98TXSF-112	-	<2	<2	-	-	-	-	-
98TXSF-113	+ F	256	16	-	-	-	-	-
98TXSF-114	+ F	>256	>256	-	-	-	-	-
98TXSF-115	+ F	>256	>256	-	-	-	-	-
98TXSF-116	+ F	>256	>256	-	-	-	-	-
98TXSF-117	+ F	>256	>256	-	-	-	-	-
98TXSF-118	-	<2	<2	-	-	-	-	-
98TXSF-119	-	<2	<2	-	-	-	-	-
98TXSF-120	+ F	>256	>256	-	-	-	-	-
Total	89/120			1/120	0/120	0/120	0/120	0/120

Table 3.9.

Table 3.10: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 7 Post-arrival at the Order-buyer Barn during the 1998 Shipping Fever Epizootic

ID of	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-1	-	<2	<2	-	-	-	-	-
98TXSF-2	-	<2	<2	-	-	-	-	-
98TXSF-3	-	<2	<2	-	-	-	-	-
98TXSF-4	+ F	>256	<2	-	-	-	-	-
98TXSF-5	-	<2	<2	-	-	-	-	-
98TXSF-6	-	<2	<2	-	-	-	-	-
98TXSF-7	+ F	>256	<2	-	-	-	-	-
98TXSF-8	+ F	>256	<2	-	-	-	-	-
98TXSF-9	+ F	>256	<2	-	-	-	-	-
98TXSF-10	+ F	>256	>256	-	-	-	-	-
98TXSF-11	+ F	>256	>256	-	-	-	-	-
98TXSF-12	+ F	>256	<2	-	-	-	-	-
98TXSF-13	+ F	>256	<2	-	-	-	-	-
98TXSF-14	+ F	>256	>256	-	-	-	-	-
98TXSF-15	+ F	>256	32	-	-	-	-	-
98TXSF-16	-	<2	<2	-	-	-	-	-
98TXSF-17	-	<2	<2	-	-	-	-	-
98TXSF-18	+ F	>256	<2	-	-	-	-	-
98TXSF-19	+ F	>256	<2	-	-	-	-	-
98TXSF-20	-	<2	<2	-	-	-	-	-
98TXSF-21	-	<2	<2	-	-	-	-	-
98TXSF-22	-	<2	<2	-	-	-	-	-
98TXSF-23	-	<2	<2	-	-	-	-	-
98TXSF-24	-	<2	<2	-	-	-	-	-
98TXSF-25	-	<2	<2	-	-	-	-	-
98TXSF-26	+ F	>256	<2	-	-	-	-	-
98TXSF-27	-	<2	<2	+	-	-	-	-
98TXSF-28	+ F	>256	<2	-	-	-	-	-
98TXSF-29	-	<2	<2	-	-	-	-	-
98TXSF-30	-	<2	<2	-	-	-	-	-
98TXSF-31	-	<2	<2	-	-	-	-	-

Table 3.10: contd.

Notes as table 3.9.

ID of	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-32	-	<2	<2	-	-	-	-	-
98TXSF-33	-	<2	<2	-	-	-	-	-
98TXSF-34	-	<2	<2	-	-	-	-	-
98TXSF-35	-	<2	<2	-	-	-	-	-
98TXSF-36	-	<2	<2	-	-	-	-	-
98TXSF-37	+ F	>256	<2	-	-	-	-	-
98TXSF-38	-	<2	<2	-	-	-	-	-
98TXSF-39	+ F	>256	<2	-	-	-	-	-
98TXSF-40	+ F	>256	<2	-	-	-	-	-
98TXSF-41	-	<2	<2	-	-	-	-	-
98TXSF-42	+ F	>256	<2	-	-	-	-	-
98TXSF-43	+ F	>256	<2	-	-	-	-	-
98TXSF-44	-	<2	<2	-	-	-	-	-
98TXSF-45	+ F	>256		-	-	-	-	-
98TXSF-46	-	<2	<2	-	-	-	-	-
98TXSF-47	-	<2	<2	-	-	-	-	-
98TXSF-48	+ F	>256	<2	-	-	-	-	-
98TXSF-49	+ F	>256	<2	-	-	-	-	-
98TXSF-50	+ F	>256	<2	-	-	-	-	-
98TXSF-51	+ F	>256		-	-	-	-	-
98TXSF-52	-	<2	<2	-	-	-	-	-
98TXSF-53	-	<2	<2	-	-	-	-	-
98TXSF-54	+ F	>256	<2	-	-	-	-	-
98TXSF-55	+ F	>256	<2	-	-	-	-	-
98TXSF-56	+ F	>256	<2	-	-	-	-	-
98TXSF-57	+ F	>256	<2	-	-	-	-	-
98TXSF-58	-	<2	<2	-	+	-	-	-
98TXSF-59	+ F	>256	<2	-	-	-	-	-
98TXSF-60	+ F	>256	<2	-	-	-	-	-
98TXSF-61	+ F	>256	<2	-	-	-	-	-
98TXSF-62	-	<2	<2	-	-	-	-	-
98TXSF-63	+ F	>256	<2	-	-	-	-	-
98TXSF-64	-	<2	<2	-	-	-	-	-
98TXSF-65	-	<2	<2	-	-	-	-	-
98TXSF-66	-	<2	<2	-	-	-	-	-
98TXSF-67	+ F	>256	<2	-	-	-	-	-
98TXSF-68	+ F	>256	<2	-	-	-	-	-

Table 3.10: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-69	+ F	>256	<2	-	-	-	-	-
98TXSF-70	-	<2	<2	-	-	-	-	-
98TXSF-71	+ F	>256	>256	-	-	-	-	-
98TXSF-72	+ F	>256	>256	-	-	-	-	-
98TXSF-73	-	<2	<2	-	-	-	-	-
98TXSF-74	-	<2	<2	-	-	-	-	-
98TXSF-75	+ F	>256	<2	-	-	-	-	-
98TXSF-76	+ F	>256	>256	-	-	-	-	-
98TXSF-77	+ F	>256	<2	-	-	-	-	-
98TXSF-78	-	<2	<2	-	-	-	-	-
98TXSF-79	+ F	>256	<2	-	-	-	-	-
98TXSF-80	+ F	>256	<2	-	-	-	-	-
98TXSF-81	-	<2	<2	-	-	-	-	-
98TXSF-82	-	<2	<2	-	-	-	-	-
98TXSF-83	+ F	>256	<2	-	-	-	-	-
98TXSF-84	-	<2	<2	-	-	-	-	-
98TXSF-85	+ F	>256 (Oct. 5)	32	-	-	-	-	-
98TXSF-86	-	<2	<2	-	-	-	-	-
98TXSF-87	-	<2	<2	-	-	-	-	-
98TXSF-88	-	<2	<2	-	-	-	-	-
98TXSF-89	+ F	>256	<2	-	-	-	-	-
98TXSF-90	+ F	>256	<2	-	-	-	-	-
98TXSF-91	+ F	>256	>256	-	-	-	-	-
98TXSF-92	+ F	>256	<2	-	-	-	-	-
98TXSF-93	-	<2	<2	-	-	-	-	-
98TXSF-94	-	<2	<2	-	-	-	-	-
98TXSF-95	-	<2	<2	-	-	-	-	-
98TXSF-96	+ F	>256	<2	-	-	-	-	-
98TXSF-97	+ F	>256	<2	-	-	-	-	-
98TXSF-98	-	<2	<2	-	-	-	-	-
98TXSF-99	+ F	>256	<2	-	-	-	-	-
98TXSF-100	+ F	>256	4	-	-	-	-	-
98TXSF-101	+ F	>256	<2	-	-	-	-	-
98TXSF-102	+ F	>256	128	-	-	-	-	-
98TXSF-103	+ F	>256	<2	-	-	-	-	-
98TXSF-104	+ F	>256	<2	-	-	-	-	-
98TXSF-105	-	<2	<2	-	-	-	-	-
98TXSF-106	+ F	>256	>256	-	-	-	-	-

Table 3.10: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-107	+ F	>256	<2	-	-	-	-	-
98TXSF-108	-	<2	<2	-	-	-	-	-
98TXSF-109	+ F	>256	<2	-	-	-	-	-
98TXSF-110	+ F	>256	>256	-	-	-	-	-
98TXSF-111	+ F	>256	<2	-	-	-	-	-
98TXSF-112	-	<2	<2	-	-	-	-	-
98TXSF-113	+ F	>256	16	-	-	-	-	-
98TXSF-114	+ F	>256	>256	-	-	-	-	-
98TXSF-115	+ F	>256	4	-	-	-	-	-
98TXSF-116	+ F	>256	8	-	-	-	-	-
98TXSF-117	+ F	>256	<2	-	-	-	-	-
98TXSF-118	-	<2	<2	-	-	-	-	-
98TXSF-119	-	<2	<2	-	-	-	-	-
98TXSF-120	+ F	>256	<2	-	-	-	-	-
Total	67/120			1/120	1/120	0/120	0/120	0/120

Table 3.10.

Therefore, 67 cattle contained RBCV in nasal secretions right after their arrived at the feed yard, when adverse clinical signs of respiratory tract diseases were still evident. At the same time, 1 cattle had BHV-1 only and 1 cattle shed PI-3 only. Of the 120 cattle, 91 cattle experienced RBCV infection during the first week of this investigation. Only 6 and 4 cattle still nasally shed RBCV on days 14 and 21 post-arrival at the OBB, respectively (Tables 3.11 and 3.12). However, on day 14 post-arrival at the OBB, of the remaining 109 cattle, 4 cattle shed BHV-1 only, 1 cattle had mixed infections of BHV-1 and RBCV, and 6 cattle became PI-3-infected. On day 21 post-arrival at the OBB, of the surviving 107 cattle, 2 cattle had BHV-1 only while 13 cattle shed PI-3, and 1 of them had simultaneously RBCV infection. Again, sequential nasal swab samples remained BRSV, cytopathogenic BVDV and bovine adenovirus-negative during the 1998 epizootic.

3.3.3 Virological Findings in Fatal Cases

The 1997 shipping fever experiment: Ten cattle died on days 7 to 11 post-arrival at the OBB that were 1 to 5 days after they arrived at the ARS feed yard. Virus isolation attempts with nasal swab samples indicated that 6 of them shed RBCV on their arrival at OBB, 3 more contracted this infection during transport (Table 3.13), and no other respiratory viruses were detected before these calves died. Further analysis of 10 pneumonic lung samples revealed that 9 were RBCV-positive, no other viruses were

Table 3.11: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 14 Post-arrival at the Order-buyer Barn during the 1998 Shipping Fever Epizootic

ID of	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-1	-	<2	<2	-	-	-	-	-
98TXSF-2	-	<2	<2	-	-	-	-	-
98TXSF-3	-	<2	<2	-	+	-	-	-
98TXSF-4	-	<2	<2	-	-	-	-	-
98TXSF-5	-	<2	<2	-	+	-	-	-
98TXSF-6	-	<2	<2	-	-	-	-	-
98TXSF-7	+ F	>256	<2	-	-	-	-	-
98TXSF-8	-	<2	<2	-	-	-	-	-
98TXSF-9	-	<2	<2	-	-	-	-	-
98TXSF-10	Died							
98TXSF-11	Died							
98TXSF-12	+ F	128	<2	-	-	-	-	-
98TXSF-13	-	<2	<2	-	-	-	-	-
98TXSF-14	Died							
98TXSF-15	-	<2	<2	+	-	-	-	-
98TXSF-16	-	<2	<2	-	-	-	-	-
98TXSF-17	-	<2	<2	-	-	-	-	-
98TXSF-18	-	<2	<2	-	-	-	-	-
98TXSF-19	-	<2	<2	-	-	-	-	-
98TXSF-20	-	<2	<2	-	-	-	-	-
98TXSF-21	-	<2	<2	-	-	-	-	-
98TXSF-22	-	<2	<2	-	-	-	-	-
98TXSF-23	-	<2	<2	-	-	-	-	-
98TXSF-24	-	<2	<2	-	-	-	-	-
98TXSF-25	-	<2	<2	-	-	-	-	-
98TXSF-26	-	<2	<2	-	-	-	-	-
98TXSF-27	-	<2	<2	-	-	-	-	-
98TXSF-28	-	<2	<2	-	-	-	-	-
98TXSF-29	-	<2	<2	-	-	-	-	-
98TXSF-30	-	<2	<2	-	-	-	-	-
98TXSF-31	-	<2	<2	-	-	-	-	-

Table 3.11: contd.

Notes as table 3.9.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-32	-	<2	<2	-	+	-	-	-
98TXSF-33	-	<2	<2	-	-	-	-	-
98TXSF-34	-	<2	<2	-	-	-	-	-
98TXSF-35	-	<2	<2	-	-	-	-	-
98TXSF-36	-	<2	<2	-	-	-	-	-
98TXSF-37	-	<2	<2	-	-	-	-	-
98TXSF-38	-	<2	<2	-	-	-	-	-
98TXSF-39	-	<2	<2	-	-	-	-	-
98TXSF-40	-	<2	<2	-	-	-	-	-
98TXSF-41	-	<2	<2	-	-	-	-	-
98TXSF-42	+ F, HC	>256	<2	-	-	-	-	-
98TXSF-43	-	<2	<2	-	-	-	-	-
98TXSF-44	-	<2	<2	-	-	-	-	-
98TXSF-45	-	<2	<2	-	-	-	-	-
98TXSF-46	-	<2	<2	-	-	-	-	-
98TXSF-47	-	<2	<2	-	-	-	-	-
98TXSF-48	-	<2	<2	-	+	-	-	-
98TXSF-49	-	<2	<2	-	-	-	-	-
98TXSF-50	-	<2	<2	-	-	-	-	-
98TXSF-51	-	<2	<2	-	-	-	-	-
98TXSF-52	-	<2	<2	-	-	-	-	-
98TXSF-53	-	<2	<2	-	-	-	-	-
98TXSF-54	-	<2	<2	-	-	-	-	-
98TXSF-55	-	<2	<2	-	-	-	-	-
98TXSF-56	-	<2	<2	-	-	-	-	-
98TXSF-57	-	<2	<2	-	+	-	-	-
98TXSF-58	-	<2	<2	-	-	-	-	-
98TXSF-59	-	<2	<2	-	-	-	-	-
98TXSF-60	-	<2	<2	-	-	-	-	-
98TXSF-61	+ F	>256	<2	-	-	-	-	-
98TXSF-62	-	<2	<2	-	+	-	-	-
98TXSF-63	-	<2	<2	-	-	-	-	-
98TXSF-64	-	<2	<2	-	-	-	-	-
98TXSF-65	-	<2	<2	-	-	-	-	-
98TXSF-66	-	<2	<2	-	-	-	-	-
98TXSF-67	-	<2	<2	-	-	-	-	-
98TXSF-68	-	<2	<2	-	-	-	-	-
98TXSF-69	-	<2	<2	-	-	-	-	-

Table 3.11: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-70	-	<2	<2	-	-	-	-	-
98TXSF-71	Died							
98TXSF-72	Died							
98TXSF-73	-	<2	<2	-	-	-	-	-
98TXSF-74	-	<2	<2	-	-	-	-	-
98TXSF-75	-	<2	<2	-	-	-	-	-
98TXSF-76	-	<2	<2	-	-	-	-	-
98TXSF-77	-	<2	<2	-	-	-	-	-
98TXSF-78	-	<2	<2	-	-	-	-	-
98TXSF-79	-	<2	<2	-	-	-	-	-
98TXSF-80	-	<2	<2	-	-	-	-	-
98TXSF-81	-	<2	<2	-	-	-	-	-
98TXSF-82	-	<2	<2	-	-	-	-	-
98TXSF-83	-	<2	<2	+	-	-	-	-
98TXSF-84	-	<2	<2	-	-	-	-	-
98TXSF-85	Died							
98TXSF-86	-	<2	<2	-	-	-	-	-
98TXSF-87	-	<2	<2	-	-	-	-	-
98TXSF-88	-	<2	<2	-	-	-	-	-
98TXSF-89	-	<2	<2	-	-	-	-	-
98TXSF-90	-	<2	<2	-	-	-	-	-
98TXSF-91	Died							
98TXSF-92	-	<2	<2	-	-	-	-	-
98TXSF-93	-	<2	<2	-	-	-	-	-
98TXSF-94	-	<2	<2	-	-	-	-	-
98TXSF-95	-	<2	<2	-	-	-	-	-
98TXSF-96	-	<2	<2	+	-	-	-	-
98TXSF-97	-	<2	<2	-	-	-	-	-
98TXSF-98	-	<2	<2	-	-	-	-	-
98TXSF-99	-	<2	<2	-	-	-	-	-
98TXSF-100	-	<2	<2	+	-	-	-	-
98TXSF-101	-	<2	<2	-	-	-	-	-
98TXSF-102	Died							
98TXSF-103	-	<2	<2	-	-	-	-	-
98TXSF-104	-	<2	<2	-	-	-	-	-
98TXSF-105	-	<2	<2	-	-	-	-	-
98TXSF-106	Died							
98TXSF-107	+ F	>256	<2	-	-	-	-	-

Table 3.11: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-108	-	<2	<2	-	-	-	-	-
98TXSF-109	-	<2	<2	-	-	-	-	-
98TXSF-110	Died							
98TXSF-111	-	<2	<2	-	-	-	-	-
98TXSF-112	-	<2	<2	-	-	-	-	-
98TXSF-113	-	<2	<2	-	-	-	-	-
98TXSF-114	Died							
98TXSF-115	+ F	>256	<2	+	-	-	-	-
98TXSF-116	-	<2	<2	-	-	-	-	-
98TXSF-117	-	<2	<2	-	-	-	-	-
98TXSF-118	-	<2	<2	-	-	-	-	-
98TXSF-119	-	<2	<2	-	-	-	-	-
98TXSF-120	-	<2	<2	-	-	-	-	-
Total	6/109			5/109	6/109	0/109	0/109	0/109

Table 3.11.

Table 3.12: Respiratory Bovine Virus Isolation from Nasal Swab Samples of Cattle On Day 21 Post-arrival at the Order-buyer Barn during the 1998 Shipping Fever Epizootic

ID of	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
Cattle	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-1	-	<2	<2	-	-	-	-	-
98TXSF-2	-	<2	<2	-	-	-	-	-
98TXSF-3	-	<2	<2	-	-	-	-	-
98TXSF-4	-	<2	<2	-	-	-	-	-
98TXSF-5	-	<2	<2	-	-	-	-	-
98TXSF-6	-	<2	<2	-	-	-	-	-
98TXSF-7	-	<2	<2	-	-	-	-	-
98TXSF-8	-	<2	<2	-	-	-	-	-
98TXSF-9	-	<2	<2	-	-	-	-	-
98TXSF-10	Died							
98TXSF-11	Died							
98TXSF-12	-	<2	<2	-	-	-	-	-
98TXSF-13	-	<2	<2	-	-	-	-	-
98TXSF-14	Died							
98TXSF-15	-	<2	<2	+	-	-	-	-
98TXSF-16	-	<2	<2	-	-	-	-	-
98TXSF-17	-	<2	<2	-	-	-	-	-
98TXSF-18	-	<2	<2	-	-	-	-	-
98TXSF-19	-	<2	<2	-	-	-	-	-
98TXSF-20	-	<2	<2	-	-	-	-	-
98TXSF-21	-	<2	<2	-	-	-	-	-
98TXSF-22	-	<2	<2	-	+	-	-	-
98TXSF-23	-	<2	<2	-	-	-	-	-
98TXSF-24	-	<2	<2	-	-	-	-	-
98TXSF-25	-	<2	<2	-	+	-	-	-
98TXSF-26	+ F	64	64	-	+	-	-	-
98TXSF-27	-	<2	<2	-	-	-	-	-
98TXSF-28	-	<2	<2	-	-	-	-	-
98TXSF-29	-	<2	<2	-	-	-	-	-
98TXSF-30	-	<2	<2	-	+	-	-	-
98TXSF-31	-	<2	<2	-	-	-	-	-

Table 3.12: contd.

Notes as table 3.9.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-32	-	<2	<2	-	+	-	-	-
98TXSF-33	-	<2	<2	-	-	-	-	-
98TXSF-34	-	<2	<2	-	-	-	-	-
98TXSF-35	-	<2	<2	-	-	-	-	-
98TXSF-36	-	<2	<2	-	-	-	-	-
98TXSF-37	-	<2	<2	-	-	-	-	-
98TXSF-38	-	<2	<2	-	-	-	-	-
98TXSF-39	-	<2	<2	-	-	-	-	-
98TXSF-40	-	<2	<2	-	-	-	-	-
98TXSF-41	-	<2	<2	-	-	-	-	-
98TXSF-42	-	<2	<2	-	+	-	-	-
98TXSF-43	+ F	16	16	-	-	-	-	-
98TXSF-44	-	<2	<2	-	-	-	-	-
98TXSF-45	+ F (3)	16, >256 (3)	16	-	-	-	-	-
98TXSF-46	-	<2	<2	-	-	-	-	-
98TXSF-47	-	<2	<2	-	-	-	-	-
98TXSF-48	-	<2	<2	-	-	-	-	-
98TXSF-49	-	<2	<2	-	-	-	-	-
98TXSF-50	-	<2	<2	-	-	-	-	-
98TXSF-51	-	<2	<2	-	-	-	-	-
98TXSF-52	-	<2	<2	-	+	-	-	-
98TXSF-53	-	<2	<2	-	-	-	-	-
98TXSF-54	-	<2	<2	-	-	-	-	-
98TXSF-55	-	<2	<2	-	-	-	-	-
98TXSF-56	-	<2	<2	-	-	-	-	-
98TXSF-57	-	<2	<2	-	-	-	-	-
98TXSF-58	-	<2	<2	-	-	-	-	-
98TXSF-59	-	<2	<2	-	-	-	-	-
98TXSF-60	-	<2	<2	-	+	-	-	-
98TXSF-61	-	<2	<2	-	-	-	-	-
98TXSF-62	-	<2	<2	-	-	-	-	-
98TXSF-63	-	<2	<2	-	-	-	-	-
98TXSF-64	-	<2	<2	-	-	-	-	-
98TXSF-65	-	<2	<2	-	-	-	-	-
98TXSF-66	-	<2	<2	-	+	-	-	-
98TXSF-67	-	<2	<2	-	-	-	-	-
98TXSF-68	-	<2	<2	-	-	-	-	-
98TXSF-69	-	<2	<2	-	-	-	-	-

Table 3.12: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-70	-	<2	<2	-	+	-	-	-
98TXSF-71	Died							
98TXSF-72	Died							
98TXSF-73	-	<2	<2	-	-	-	-	-
98TXSF-74	-	<2	<2	-	-	-	-	-
98TXSF-75	-	<2	<2	-	-	-	-	-
98TXSF-76	-	<2	<2	-	-	-	-	-
98TXSF-77	-	<2	<2	-	-	-	-	-
98TXSF-78	-	<2	<2	-	-	-	-	-
98TXSF-79	-	<2	<2	-	-	-	-	-
98TXSF-80	-	<2	<2	-	-	-	-	-
98TXSF-81	-	<2	<2	-	-	-	-	-
98TXSF-82	-	<2	<2	-	+	-	-	-
98TXSF-83	-	<2	<2	-	-	-	-	-
98TXSF-84	-	<2	<2	-	-	-	-	-
98TXSF-85	Died							
98TXSF-86	-	<2	<2	-	-	-	-	-
98TXSF-87	-	<2	<2	-	-	-	-	-
98TXSF-88	-	<2	<2	-	-	-	-	-
98TXSF-89	-	<2	<2	-	-	-	-	-
98TXSF-90	-	<2	<2	-	-	-	-	-
98TXSF-91	Died							
98TXSF-92	-	<2	<2	-	-	-	-	-
98TXSF-93	-	<2	<2	-	+	-	-	-
98TXSF-94	-	<2	<2	-	-	-	-	-
98TXSF-95	-	<2	<2	-	-	-	-	-
98TXSF-96	-	<2	<2	-	-	-	-	-
98TXSF-97	-	<2	<2	-	-	-	-	-
98TXSF-98	-	<2	<2	-	-	-	-	-
98TXSF-99	-	<2	<2	-	-	-	-	-
98TXSF-100	-	<2	<2	+	-	-	-	-
98TXSF-101	-	<2	<2	-	-	-	-	-
98TXSF-102	Died							
98TXSF-103	+ F	64	<2	-	-	-	-	-
98TXSF-104	Died							
98TXSF-105	-	<2	<2	-	-	-	-	-
98TXSF-106	Died							
98TXSF-107	-	<2	<2	-	-	-	-	-

Table 3.12: contd.

ID of Cattle	G Clone Cell Dependent- RBCV			BT or GBK Cell Dependent-Viruses				
	CPE	HA Titer	RDE Titer	BHV-1	PI-3	cpBVDV	BAV	BRSV
98TXSF-108	-	<2	<2	-	-	-	-	-
98TXSF-109	-	<2	<2	-	-	-	-	-
98TXSF-110	Died							
98TXSF-111	-	<2	<2	-	-	-	-	-
98TXSF-112	-	<2	<2	-	-	-	-	-
98TXSF-113	-	<2	<2	-	-	-	-	-
98TXSF-114	Died							
98TXSF-115	Died							
98TXSF-116	-	<2	<2	-	-	-	-	-
98TXSF-117	-	<2	<2	-	-	-	-	-
98TXSF-118	-	<2	<2	-	+	-	-	-
98TXSF-119	-	<2	<2	-	-	-	-	-
98TXSF-120	-	<2	<2	-	-	-	-	-
Total	4/107			2/107	13/107	0/107	0/107	0/107

Table 3.12.

Table 3.13: Isolation of Respiratory Bovine Viruses and *Pasteurella spp.* from Nasal and Lung Samples of Cattle that Died in the 1997 Shipping Fever Epizootic

ID of Cattle	Death on Day X	RBCV/OV Isolated from Nasal Samples		Lung Samples	
		OBB	Day 7	RBCV/OV	*Pasteurella spp.*
97TXSF-52	7	-/-	+/-	+/-	PhA1, Pm
97TXSF-5	8	+/-	+/-	+/-	PhA1
97TXSF-15	8	+/-	+/-	+/-	PhA6
97TXSF-19	8	+/-	+/-	+/-	PhA1
97TXSF-43	8	+/-	+/-	+/-	PhA1
97TXSF-62	8	-/-	+/-	+/-	PhA1
97TXSF-75	8	-/-	+/-	+/-	PhA1
97TXSF-96	8	+/-	+/-	+/-	PhA1
97TXSF-58	9	+/-	+/-	+/-	PhA1
97TXSF-63	11	-/-	-/-	-/-	PhA1
Total		6/0	9/0	9/0	10

Samples were supplied by Dr. Purdy at United States Department of Agriculture-Agriculture Research Service in Bushland, Texas. 97TXSF: nasal swab/lung samples collected from cattle under investigation of shipping fever (SF) pneumonia at Texas (TX) in 1997 (97); Day X: X days post-arrival at the order-buyer barn; OBB: on arrival at the order-buyer barn; RBCV: respiratory bovine coronavirus; OV: other viruses; +: virus was isolated; -: virus was not isolated; PhA1: *Pasteurella haemolytica* type A1; PhA6: *Pasteurella haemolytica* type A6; Pm: *Pasteurella multocida.*

isolated. The RBCV plaque forming units (PFU) per gram of lung tissues ranged from 8.0×10^3 to 5.0×10^6 (Table 3.14).

The 1998 shipping fever experiment: Sixteen cattle died on days 7 to 38 post-arrival at the OBB that were 1 to 32 days after they arrived at the ARS feed yard. Fifteen of them nasally shed RBCV when they arrived at the OBB, and all of them did so after they arrived at the ARS feed yard (Table 3.15). Again, no other respiratory bovine viruses were detected from nasal samples during the 1st week of experiment. On day 14, of the remaining 5 cattle, 1 calf was nasally both RBCV- and BHV-1-positive, and 2 had BHV-1 infection only. On day 21, of the 3 survived cattle, 2 shed BHV-1, and 1 of them continued to shed BHV-1 along with PI-3 a week later. On day 35, only 1 calf was left, nasally shedding PI-3 only. Virus isolation on 15 available lung samples indicated that 9 lung samples from cattle that died between days 7 and 9 post-arrival at the OBB were RBCV-positive, while the lung samples from the remaining cattle that died between days 13 to 38 days post-arrival at the OBB were RBCV-negative, but BHV-1 and PI-3 were detected once and twice. Lung tissues did not yield BRSV, cytopathogenic BVDV, or bovine adenovirus. The RBCV plaque-forming units (PFU) per gram of lung tissues ranged from 1.0×10^3 to 1.2×10^7 (Table 3.16).

The 1998 winter pasture pneumonia: Of the 3 cattle that died of acute winter pasture pneumonia in the Northwest Louisiana during 1998, RBCV were isolated from

Table 3.14: Loads of Respiratory Bovine Coronaviruses and *Pasteurella spp.* in Lungs of Cattle that Died in the 1997 Shipping Fever Epizootic

ID of Cattle	Death on Day X	RBCV (PFU/g)	*Pasteurella spp.* (CFU/g)
97TXSF-52	7	5.0×10^6	1.0×10^6(a), 1.6×10^7(b)
97TXSF-5	8	1.2×10^5	7.0×10^8
97TXSF-15	8	3.0×10^6	4.8×10^8
97TXSF-19	8	8.0×10^4	1.4×10^9
97TXSF-43	8	8.0×10^3	3.4×10^8
97TXSF-62	8	1.0×10^5	8.2×10^8
97TXSF-75	8	1.4×10^6	1.5×10^7
97TXSF-96	8	3.4×10^6	1.1×10^7
97TXSF-58	9	2.0×10^5	1.9×10^6
97TXSF-63	11	-	2.8×10^6

PFU/g: plaque forming units per gram of lung tissue; CFU/g: colony forming units per gram of lung tissue; (a): for PhA1; (b): for Pm; other notes as table 3.13.

Table 3.15: Isolation of Respiratory Bovine Viruses and *Pasterurella spp.* from Nasal and Lung Samples of Cattle that Died in the 1998 Shipping Fever Epizootic

ID of Cattle	Death on Day X	RBCV/OV isolated from nasal samples						Lung samples	
		OBB	Day 7	Day 14	Day 21	Day 28	Day 35	RBCV/OV	*Pasteurella spp.*
98TXSF-85*	7	-/-	+/-					+/-	Pm
98TXSF-10	8	+/-	+/-					+/-	PhA1, Pm
98TXSF-11	8	+/-	+/-					+/-	PhA1
98TXSF-14	8	+/-	+/-					+/-	Pm
98TXSF-91	8	+/-	+/-					+/-	PhA1, Pm
98TXSF-110	8	+/-	+/-					+/-	Pm
98TXSF-71	9	+/-	+/-					+/-	PhA1, Pm
98TXSF-72**	9	+/-	+/-					+/-	PhA1, Pm
98TXSF-106**	9	+/-	+/-					+/-	PhA1
98TXSF-114**	9	+/-	+/-					NA/NA	PhA1
98TXSF-102**	13	+/-	+/-					-/-	PhA2
98TXSF-104**	15	+/-	+/-	-/-				-/-	PhA1
98TXSF-115**	16	+/-	+/-	+/BHV-1				-/BHV-1	PhA1
98TXSF-36**	29	+/-	+/-	-/-	-/-			-/-	Pm
98TXSF-15**	33	+/-	+/-	-/BHV-1	-/BHV-1	PI-3/BHV-1		-/PI-3	-
98TXSF-100**	38	+/-	+/-	-/BHV-1	-/BHV-1	-/-	-/PI-3	-/PI-3	Pm

Samples were supplied by Dr. Purdy at United States Department of Agriculture-Agriculture Research Service in Bushland, Texas. 98TXSF: nasal swab/lung samples collected from cattle under investigation of shipping fever (SF) pneumonia at Texas (TX) in 1998 (98); *: Calves with odd numbers were vaccinated with an experimental bacterin-toxoid of *P. haemolytica;* **: Calves with 40°C or higher temperatures were treated with Micotif or Liquamycin LA200; Other notes as table 3.13.

Table 3.16: Loads of Respiratory Bovine Coronaviruses and *Pasteurella spp.* in Lungs of Cattle that Died in the 1998 Shipping Fever Epizootic

ID of Cattle	Death on Day X	RBCV (PFU/g)	*Pasteurella spp.* (CFU/g)
98TXSF-85*	7	6.0×10^4	6.0×10^5
98TXSF-10	8	1.0×10^5	3.3×10^8 (a), 2.5×10^6 (b)
98TXSF-11	8	6.0×10^6	6.3×10^8
98TXSF-14	8	1.0×10^3	2.4×10^8
98TXSF-91	8	1.2×10^7	4.0×10^7 (a), 1.4×10^8 (b)
98TXSF-110	8	2.4×10^6	2.3×10^9
98TXSF-71	9	4.0×10^5	3.0×10^8 (a), 4.1×10^8 (b)
98TXSF-72**	9	2.0×10^4	3.1×10^8 (a), 2.1×10^8 (b)
98TXSF-106**	9	2.0×10^4	2.5×10^6
98TXSF-114**	9	NA/NA	8.0×10^6
98TXSF-102**	13		9.5×10^5
98TXSF-104**	15		2.9×10^6
98TXSF-115**	16		4.0×10^5
98TXSF-36**	29		1.1×10^8
98TXSF-15**	33		-
98TXSF-100**	38		7.5×10^7

Notes as tables 3.14 and 3.15.

both trachea and lung samples, and, again, no other respiratory bovine viruses were detected (Table 3.17).

3.3.4 Bacteriological Findings in Fatal Cases

The 1997 shipping fever experiment: *Pasteurella haemolytica* was isolated from all 10 lung samples (Table 3.13). One calf had a pure culture of *P. haemolytica* A6, while the other 9 calves had *P. haemolytica* A1 infetions, and 1 of them also had *P. multocida* infection. Loads of *Pasterurella spp.* ranged from 1.0×10^6 to 1.4×10^9 colony forming units (CFU) per gram of lung tissue (Table 3.14).

The 1998 shipping fever experiment: *Pasteurella spp.* was cultivated from 15 of the 16 lung samples (Table 3.15). Five calf had only *P. multocida* infection, 1 calf had only *P. haemolytica* A2 isolate in the lung, and 4 calves had mixed infections of *P. haemolytica* A1 and *P. multocida.* The bacterial isolates from the remaining 5 lung samples were identified as *P. haemolytica* A1. Loads of *Pasterurella spp.* ranged from 4.0×10^5 to 2.3×10^9 CFU per gram of lung tissue (Table 3.16).

The 1998 winter pasture pneumonia: Interestingly, no *Pasterurella spp.* were detected from either lung or trachea samples of 3 cattle that died of winter pasture pneumonia (Table 3.17).

3.3.5 Correlation between RBCV Infection and Respiratory Tract Diseases

A correlation existed between the number of cattle which developed respiratory tract diseases, including fatal outcomes, and frequency of RBCV detection as presented

Table 3.17: Isolation of Respiratory Bovine Viruses and *Pasterurella spp.* from Trachea and Lung Samples of Cattle that Died in the Winter Pasture

ID of Samples	RBCV	Other Viruses	*Pasteurella spp.*
985987-trachea	+ (64 HAU)	-	-
985987-lung	+ (64 HAU)	-	-
880-trachea	+ (>256 HAU)	-	-
880-lung	+ (>256 HAU)	-	-
881-trachea	+ (>256 HAU)	-	-
881-lung	+ (>256 HAU)	-	-

Samples were provided by Dr. Jeffrey Anderson; +: infectious agents were detected; -: no infectious agents were detected; HAU: hemagglutinin units; other notes as table 3.13.

in Table 3.18 and 3.19. The RBCV was successfully isolated from 81 of the 93 clinically sick or dead cattle in the 1997 investigation, while the 1998 experiment included 89 RBCV-positive cattle of the 107 sick or dead ones. Twelve and 18 cattle exhibited adverse clinical signs of respiratory tract disease but did not shed RBCV during the 1997 and 1998 epizootics, respectively. Five cattle of the 1997 and 2 cattle of the 1998 had RBCV infections but remained clinically normal. Seven and 11 cattle were RBCV isolation-negative, and had no adverse respiratory signs of disease throughout the 1997 and 1998 experiment periods, respectively. Statistically, the sensitivity and specificity of RBCV isolations compared with respiratory tract diseases of cattle were 87% and 58%, respectively, for the 1997 epizootic, and 83% and 85%, respectively, for the 1998 experiment.

3.4 Discussion

During 1993 and 1998, we investigated 388 cattle from 11 different states. A viral agent exclusively multiplying in G clone cell cultures was successfully isolated from nasal swab samples of 225 cattle and from trachea and lung samples of 20 cattle during the early phase of respiratory tract infections (Tables 3.20 and 3.21). Investigated cattle included those with acute respiratory distress after shipping, during shipping fever epizootic, in a livestock show, and from stocker cattle on winter pastures in the Southern U.S.A. This viral agent was identified as coronavirus and is referred to as

Table 3.18: Correlation between Respiratory Bovine Coronavirus Infection and Signs of Respiratory Tact Diseases of Cattle in the 1997 Shipping Fever Epizootic.

Isolation of RBCV	Sick or Dead	Clinical Normal	Total
RBCV(+)	81	5	86
RBCV(-)	12	7	19
Total	93	12	105

RBCV(+): respiratory bovine coronavirus isolated in the G clone cells; RBCV(-): respiratory bovine coronavirus not isolated in the G clone cells; other notes as table 3.13.

Table 3.19: Correlation between Respiratory Bovine Coronavirus Infection and Signs of Respiratory Tact Diseases of Cattle in the 1998 Shipping Fever Epizootic.

Isolation of RBCV	Sick or Dead	Clinical Normal	Total
RBCV(+)	89	2	91
RBCV(-)	18	11	29
Total	107	13	120

Notes as table 3.18.

Table 3.20: Frequency of Virus Isolations from Nasal Secretions of Cattle with Acute Respiratory Tract Diseases

	Viruses Detected in Nasal Swab Samples					
Disease Episodes	**RBCV**	**BHV-1**	**PI-3**	**cpBVDV**	**BAV**	**BRSV**
1993 Kansas feedyard	32/50	0/50	0/50	0/50	0/50	0/50
1993 Arizona feedyard	6/50	0/50	4/50	0/50	0/50	0/50
1997 TXSF epizootic	86/105	7/105	4/105	0/105	0/105	0/105
1998 TXSF epizootic	91/120	7/120	19/120	0/120	0/120	0/120
LSU livestock show	4/29	2/29	0/29	0/29	1/29	0/29
Hospital cases	2/16	1/16	0/16	0/16	0/16	0/16
Winter pastures	4/15	1/15	0/15	0/15	0/15	0/15
Positive/total	225/385	18/385	27/385	0/385	1/385	0/385

Notes as tables 3.1, 3.5, and 3.9.

Table 3.21: Frequency of Virus Isolations from Lungs of Cattle with Fatal Pneumonia

	Viruses Detected in Lung Samples					
Disease Episodes	**RBCV**	**BHV-1**	**PI-3**	**cpBVDV**	**BAV**	**BRSV**
1997 TXSF epizootic	9/10	0/10	0/10	0/10	0/10	0/10
1998 TXSF epizootic	8/15	1/15	2/15	0/15	0/15	0/15
Winter pasture pneumonia	3/3	0/3	0/3	0/3	0/3	0/3
Positive/total	20/28	1/28	2/28	0/28	0/28	0/28

Notes as tables 3.1, 3.5, and 3.9.

RBCV on the basis of site of isolation, clinical signs of affected cattle, presence of HA and specific receptor-destroying functions, cell fusion and other cytopathic properties in G clone cells as well as ultrastructural features. The use of G clone cells, which has a homogeneous epithelial cell population, played an important role in the isolation of RBCV. The parent HRT-18 cell cultures have properties of polarized epithelial cells, and contain a rather heterogeneous cell population (Payne and Storz, 1990; Tompkins et al., 1974).

The RBCV isolates have unique properties that distinguished them from wild-type, hemagglutinating EBCV. Distinguishing features are: (1) The RBCV were isolated in the first G clone cell passage without trypsin enhancement. Trypsin activation was required for the isolation of EBCV (Storz et al., 1981). (2) Thc RBCV have unusually high cell-fusing activities for the G clone cells. (3) The RBCV have a restricted hemagglutination pattern. They agglutinate only mouse and rat, but not chicken and bovine RBC. (4) Comparison of nucleotide and predicted amino acid sequences of all genes encoded by the 3' genomic portion (9.5kb) of RBCV and EBCV defined pinpoint RBCV-specific mutations that are suggested to be associated with pneumopathogenicity and other features of RBCV (Chouljenko et al., 1998a and b). (5) The hemagglutinin-esterase of selected wild-type RBCV and EBCV strains differed in AE kinetic activities at 37°C and 39°C (Lin et al., 1997a). The high degree of nucleotide conservation among all RBCV isolates indicated that all these viruses evolved from a common prototypic

ancestral genome (Chouljenko et al., 1998a; Storz et al., 2000a). The dynamic evolution of new viral genotypes containing single nucleotide changes at specific genomic loci was evident within the same calf at different time points of disease progression (Chouljenko et al., 1998a; Storz et al., 2000a).

The high rate of RBCV isolation and virtual absence of other respiratory virus infections during the early stages of the investigated respiratory tract disease epizootics indicated that RBCV played a causative role in shipping fever pneumonia and winter pasture pneumonia. Although serological and antigen detection tests identified a corona-like agent as a potential cause of respiratory disease of cattle, isolation of wild-type RBCV had not been successful (Appel et al., 1992; Carman and Hazlett, 1992; Heckert et al., 1991; Herbst et al., 1989; Jimenez et al., 1989; Möstl and Bürki, 1988). Remarkably, the involvement of RBCV in epizootics of shipping fever pneumonia was not suspected in the past (Yates, 1982).

The novel virus isolation techniques allow us to exclude other respiratory bovine viruses as major viral causes associated with these episodes of acute respiratory tract diseases. Almost all the investigated cattle had been vaccinated with a modified-live virus vaccine containing BHV-1 and PI-3. Successful vaccination programs theoretically reduce the frequency of the respective infections. These BHV-1 or PI-3 vaccinations did not have a modulating influence on the pathogenesis of RBCV infections. The surprisingly low rate of BHV-1, PI-3, and other respiratory viruses

among these vaccinated cattle contrasts with the high frequency of RBCV isolations in these respiratory tract disease episodes under investigation (Tables 3.20 and 3.21). This finding may suggest that RBCV is establishing itself as a currently prevailing respiratory virus in the cattle population because of the immune pressure of successful modern vaccination programs, which are reducing infection with other known viral causes of BRDC.

The predominant bacterial species cultivated from pneumonic lung samples of dead cattle were *P. haemolytica*. The upper respiratory tracts but not lungs of clinically normal cattle may harbor *P. haemolytica* and other bacteria considered as potential contributing causes of shipping fever (Briggs et al., 1998; Frank et al., 1986; Frank and Briggs, 1992; Frank and Smith, 1983; Magwood et al., 1969). Certainly, *P. haemolytica* infections played a role in the pathogenesis of the severe pneumonia of the calves whose respiratory tract defenses were weakened by the high level of RBCV infection during the early phase of respiratory tract disease episodes of our investigations. The HE glycoprotein of BCV binds to the 9-O-acetylated neuraminic acid residues of glycoproteins or glycolipids on the surfaces of erythrocytes and susceptible cells, which is considered to be the major receptor determinant of coronavirus (Herrler et al., 1985; Herrler et al., 1991; Vlasak et al., 1988a and b). The AE activity of HE hydrolyzes an ester bond to cleave an acetyl group from the 9-O-position of the substrates, potentially eluting adsorbed virions and destroying the HE-binding activity of the glycans on the

cell membrane. The process of binding to 9-O-acetylated receptors, followed by cleavage and rebinding to intact receptors, could theoretically result in virus migration through a mucus layer and weakness of mucosal resistance barrier and thereby facilitate not only the virus infection, release and spread but also adhesion of *P. haemolytica.*

It is a widely accepted hypothesis that bovine shipping fever pneumonia is resulting from multiple causes such as stressful conditions of crowding and transport and viral and bacterial infections (Hoerlein, 1980; Yates, 1982). Numerous repeated attempts to reproduce typical shipping fever pneumonia with stress alone, with virus infections alone, or with bacterial infections alone were unsuccessful, unless *P. haemolytica* infections were introduced directly into the lungs (Baldwin et al., 1967; Briggs et al., 1998; Collier, 1968; Friend et al., 1977; Hoerlein et al., 1961; Whitely et al., 1992; Yates, 1982). Stressful conditions are usually created at weaning, under auction conditions, during transport and adjustment to the feedlot environment. Identification of etiological infectious agents and their mechanisms of pathogenesis had been studied for decades. However, shipping fever pneumonia, a fatal acute respiratory tract disease, is still particularly prevalent among 6- to 8-month-old beef cattle after transport and entry into feed yards (Hoerlein, 1980; Martin, 1985; Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b; Yates, 1982).

CHAPTER 4

ANTIBODY RESPONSES TO RESPIRATORY CORONAVIRUS INFECTIONS OF CATTLE DURING SHIPPING FEVER PATHOGENESIS

4.1 Introduction

Coronaviruses, a genus in the family *Coronaviridae*, are enveloped positive-strand RNA viruses that emerge as increasingly important causes of human and animal diseases (Lai, 1990). These diseases include respiratory infections, gastroenteritis, hepatic and neurological disorders, immune-mediated disease such as feline infectious peritonitis, and persistent infections (Lai, 1990; Spaan et al., 1988; Wege et al., 1982). Bovine coronavirus (BCV) is the second most common cause of virus-induced severe enteritis or occasional pneumoenteritis in calves, and is referred to here as enteropathogenic BCV (EBCV) (Clark, 1993; Mebus et al., 1973). Winter dysentery in adult dairy cattle was also attributed to EBCV (Clark, 1993; Saif et al., 1988).

Recently, high rates of coronavirus infections were detected in respiratory tract samples of cattle with acute respiratory distress including shipping fever pneumonia (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). Shipping fever pneumonia is an acute respiratory tract disease, particularly prevalent among 6- to 8-month-old cattle after transport and entry into feed yards in Northern America (Hoerlein, 1980; Yates, 1982). The role of respiratory bovine coronavirus (RBCV) in

shipping fever pneumonia was previously not recognized. A refined virus isolation scheme was applied in recent etiological investigations. It included the G clone of human rectal tumor-18 (HRT-18) cells and Georgia bovine kidney (GBK) and bovine turbinate (BT) cells with specific permissiveness for currently known respiratory viruses of cattle including RBCV (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). This approach led to the first successful isolation of wild-type RBCV at high rates from nasal swab samples of cattle arriving at feed yards with respiratory distress, and provided the initial evidence of a potential etiological role of RBCV in shipping fever pneumonia.

The genome of EBCV consists of a single, positive-stranded RNA molecule of about 31 kilobases (kb) (Clark, 1993; Lai, 1990; Spaan et al., 1988; Wege et al., 1982). The 3' end of the genomic RNA consists of approximately 9.5kb and contains the genes for 5 structural proteins: (1) a longer peplomeric 190 kilodalton (kDa) spike glycoprotein (S) with a proteolytic cleavage site where proteases split S into 110-kDa S1 and 100-kDa S2 subunits, (2) a short peplomeric 140-kDa hemagglutinin-esterase glycoprotein (HE) which is a disulfide-linked dimer of 2 identical 65-kDa subunits, (3) a 26-kDa integral membrane glycoprotein (M), (4) a 9.5-kDa envelope protein (E), and (5) an internal phosphorylated 50-kDa nucleocapsid protein (N) (Brown and Brierly, 1995; Deregt et al., 1987; Hogue et al., 1989). Comparative nucleotide and amino acid sequence analysis of the 3' genomic portion (9.5kb) of wild-type RBCV and EBCV

strains revealed that RBCV-specific nucleotide and amino acid changes were disproportionally concentrated within the HE gene, the S gene and the genomic region between the S and E genes (Chouljenko et al., 1998b).

Immunoglobulin (Ig) G predominates over other classes in serum of cattle and accounts for around 90% of the total serum Ig (Duncan et al., 1972; McGuire et al., 1976; Williams and Spooner; 1975). The 2 major subclasses of IgG in cattle are IgG1 and IgG2 (Butler, 1969). Although IgG1 and IgG2 occur in relatively equal amounts in serum and secretions, IgG1 is the predominant isotype in colostrum and milk instead. Bovine IgG1 and IgG2 fix bovine complement, but IgG2 does not bind heterologous complement (McGuire et al., 1979; McGuire and Musoke, 1980). The antibody responses to EBCV structural proteins were first studied by Heckert et al (Heckert et al., 1991) in experimentally-exposed, colostrum-deprived, 20- to 30-hour-old calves that were inoculated orally and intranasally at birth and challenge exposed 3 weeks after inoculation. About 2 to 3 days postinoculation, these calves developed enteric disease signs, and virus shedding was detected in their feces and nasal swab samples for 4 to 9 days and 6 to 9 days, respectively. These calves first developed serum IgM to N and HE at postinoculation week (PIW) 1, and then they responded with IgG1 directed to N and S, and with moderate and slower IgG2 response to N and S antigens at PIW 2 and 3. After challenge of the immunity of these calves by EBCV inoculation, virus shedding was not detected, and calves did not become ill. An increase in IgM to N, in IgG1

antibody reactions to S, HE, M and N, and in IgG2 responses to S and HE was detected. The S and HE antigens elicited virus infectivity-neutralizing antibodies.

Previously, extensive investigations on the kinetics of antibody responses of cattle to respiratory viruses other than RBCV demonstrated a crucial role of Ig isotypes in disease development and protection (Hoerlein, 1980; Kimman et al., 1989a and b; LeJan and Asso, 1980; Potgieter, 1975; Rossi and Kiesel, 1976; Yates, 1982). We hypothesized that differences in either immune response to RBCV or RBCV-specificity of antibody reaction would effect the clinical outcome of shipping fever pneumonia. Consequently, we monitored these parameters of RBCV infections during a severe epizootic of shipping fever pneumonia which was prospectively designed, and included sequential examinations and samplings. These cattle were studied clinically, virologically and immunologically from the initial phases of infection to recovery or fatal outcomes. The investigation facilitated the first comprehensive assessment of currently prevailing respiratory virus infections of market-stressed cattle during a naturally occurring epizootic. A surprisingly high rate of RBCV infections in the virtual absence of other respiratory bovine viruses was detected in the early stage of this epizootic (Storz et al., 1999; Storz et al., 2000a and b). The specific objectives of this investigation were to assess the kinetics of total antibody responses of immunologically mature cattle to RBCV infections during a naturally occurring epizootic of shipping fever pneumonia, to define the immunoisotype responses and the antigenic reactivities

of the S, HE, M, N structural proteins of RBCV, and to relate these findings with isolation of RBCV from nasal swab and lung samples and development of respiratory tract disease.

4.2 Materials and Methods

4.2.1 Experimental Design

One hundred and five 6- to 8-month-old cattle were included in this naturally evolving and prospectively monitored epizootic which occurred in 1997. The mixed-breed cattle were assembled on day 0 at an order-buyer barn (OBB), identified by ear tags and clinically examined. Nasal swab and blood samples were collected, followed by vaccination with commercially available modified-live vaccines against BHV-1 and PI-3 (Prevail, Rhone Merieux Inc., Athens, Ga.), and a 7-way clostridial vaccine (Electroid 7, Mallinckrodt Veterinary Inc., Mundelein, Il.). After a stay at OBB, the cattle were transported 1932 kilometers to the feed yard jointly operated by the Agricultural Research Service and the Texas Agricultural Experimental Station in Bushland, Texas. Nasal swab samples were taken on days 7, 14, and 21, and blood for serum harvest was collected on days 7, 14, 21, 28 and 35.

Clinical signs of respiratory tract diseases and results of RBCV isolation assigned these cattle into 5 response groups based on results reported elsewhere (Table 4.1) (Storz et al., 1999; Storz et al., 2000a and b). Response group 1 included 72 cattle that exhibited clinical signs of respiratory tract disease, and were shedding RBCV on day 0,

day 7 or both. Seven animals were randomly chosen from each shedding pattern for testing in this study. Response group 2 contained 5 test cattle that secreted RBCV in nasal discharges without adverse respiratory signs. The 10 cattle of response group 3 developed severe pneumonia, and died on days 7 to 11, and 9 that nasally shed RBCV were selected. Eighteen cattle remained RBCV isolation-negative. Eleven of them were included in response group 4 because they had fever and other respiratory signs, while the remaining 7 calves (response group 5) maintained clinically healthy during the 5-week investigation. Samples of 7 representative cattle from response groups 4 and 5 were serologically analyzed. Test results of 49 cattle on sequential serum samples were included in this report.

4.2.2 Cell Line and Virus Isolate

A wild-type strain of RBCV (RBCV-97TXSF-Lu15-2) was isolated from the lung tissue of calf 97TXSF-15 as described (Storz et al., 1999; Storz et al., 2000a and b), propagated in the G clone of HRT-18 cells, and used at its 2nd passage for antigen preparation.

4.2.3 Virus Purification

Virus purification was performed according to Zhang et al. (Zhang et al., 1994). Infected G clone cell cultures with approximately 90% of the cells showing cytopathic expression were subjected to 3 cycles of freezing and thawing, and sonicating 4 times for 15 s at a power setting of 4 (Branson Sonifier cell disruptor 200, Branson

Table 4.1: Respiratory Coronavirus Isolations and Clinical Signs of Cattle in Response Groups of the 1997 Shipping Fever Epizootic

Response Group	No. of Cattle	No. of RBCV Isolation-Positive Cattle				Clinical Signs		No. of Ab-Tested Cattle
		Day 0	Day 7	Day 14	Day 21	RTD	Death	
1a	12	12	0	1	0	Yes	No	7
1b	44	44	44	2	3	Yes	No	7
1c	16	0	16	1	1	Yes	No	7
2	5	2	3	1	0	No	No	5
3	10*	6	9			Yes	Yes	9
4	11	0	0	0	0	Yes	No	7
5	7	0	0	0	0	No	No	7
Total	105	64	72	5	4			49

RBCV: respiratory bovine coronavirus; RTD: clinical signs of respiratory tract disease; *cattle died on days 7 to 11.

Ultrasonics Co, Danbury, Conn.), and centrifugation at 1,500 × g for 30 min. Supernatant fluids were collected, and precipitated overnight at 4°C with 10% (w/v) polyethylene glycol 8,000 and 0.5 M NaCl in TNE buffer (100 mM Tris, 10 mM NaCl, 1 mM EDTA pH 7.4), and harvested by centrifugation at 1,500 × g. Suspensions of precipitates were loaded onto a 20% sucrose cushion prepared in TNE buffer, centrifuged at 90,000 × g for 2 h. The sediments were collected and purified by centrifugation at 200,000 × g for 16 h through a 20-60% sucrose gradient prepared in TNE buffer. Isopycnic bands were collected, and the sucrose was removed through TNE buffer dilution and centrifugation at 200,000 × g for 1.5 h. The purified virus preparations were resuspended in TNE buffer and stored at –70°C.

4.2.4 Antibody Detection Using Indirect Enzyme-linked Immunosorbent Assay (ELISA)

The purified RBCV-97TXSF-Lu15-2 stock was diluted to 1 μg protein/ml in 0.1 M carbonate buffer, pH 9.6, and used to coat Microtitration ELISA plates (Immulon-2, Fisher Scientific, Pittsburgh, Pa.). The coated plates were held overnight at 4°C, and blocked for 2 h at room temperature with NET buffer (0.15 M NaCl, 1 mM EDTA, 0.05 M Tris, pH 7.4) containing 1% (w/v) bovine serum albumin (BSA) and 0.2% (v/v) NaN_3. Plates were washed 5 times with NET buffer containing 0.05% (v/v) Tween-20 prior to addition of each reagent (100 μl/well). All the reagents were added at 100 μl/well. Serum samples diluted 1:50 in NET buffer containing 1% BSA were added in

triplicate to appropriate wells. Serum 1745 (Storz and Rott, 1980; Storz and Rott, 1981) was included as positive control in the test while RBCV-antibody free serum from a normal calf (Storz and Rott, 1980; Storz and Rott, 1981) was used as negative control. Plates were incubated for 30 min at room temperature and washed as described above. Horseradish peroxidase (HRPO) conjugated, affinity-purified goat anti-bovine IgG (H+L) (Jackson Immunoresearch Inc., Avondale, Pa.) diluted at 1:20,000 was added for total immunoglobulin detection. A 1:400 dilution of HRPO-conjugated sheep anti-bovine IgM, a 1:30,000 dilution of HRPO-conjugated sheep anti-bovine IgG1, and a 1:4,000 dilution of HRPO-conjugated sheep anti-bovine IgG2 (Bethyl Laboratories Inc., Montgomery, Tx.) were used for IgM, IgG1 and IgG2 isotype quantitation, respectively. Plates were incubated for another 30 min and washed as described above. The substrate solution containing H_2O_2 and the chromogen 3,3',5,5'-tetramethylbenzidine (Kirkegarrd & Perry Laboratories Inc., Gaithersburg, Md.) was added. Plates were incubated for 5 min, and then reactions were stopped by addition of 100 µl of 0.10 M H_2SO_4. The optical density (OD) was measured with an ELISA plate reader (Dynatech MR 5000, Dynatech Laboratories Inc., Chantilly, Va.) at 450 nm. The OD_{450} values of triplicate wells were averaged for each test serum.

4.2.5 Statistical Assessments

The kinetics of total and isotype-specific antibody responses in each response group are presented as means ± standard error of the mean (SEM) of OD_{450} values. The

antibody responses were compared by an analysis of variance of repeated measures designed with a split-plot arrangement of treatments. Pairwise comparisons of treatment and day differences were conducted with Scheffe's test. Interaction effects were examined with pairwise t-tests of least square means for pre-planned comparisons of treatments at specific day levels. All tests were considered significant at a probability of $P < 0.05$.

4.2.6 Immunoblot Assays

Purified RBCV-97TXSF-Lu15-2 (1 mg protein/ml) at a volume of 250 µl was mixed with an equal volume of 2× sample buffer (0.125 M Tris, 20% [v/v] glycerol, 10% [v/v] 2-Mercaptoethanol, 4.6% [w/v] SDS) and heated to 100°C for 5 min. A 500-µl volume of this virus preparation was separated by electrophoresis in a 12% (w/v) polyacrylamide mini-gel (BioRad Laboratories, Richmond, Calif.) at 100 volts for 60 to 75 min. Proteins in the polyacrylamide gel were then electrophoretically transferred to pure nitrocellulose protein transfer membranes (Schleicher & Schuell, Keene, Nh.) at 100 volts for 90 min using an electrophoretic transfer cell (Mini-Trans-Blot, BioRad Laboratories). Blotted membranes were then blocked overnight at 4°C with 10% (w/v) nonfat-dry milk in NET buffer. The blot was mounted in a 28 chamber miniblotter apparatus (Miniblotter 28; Immunetics, Cambridge, Mass.). Twenty serum samples diluted 1:5 were tested on each blot in separate lanes for 1 h at room temperature.

Again, serum 1745 and RBCV-antibody free serum from a normal calf were included as positive and negative controls, respectively (Storz and Rott, 1980; Storz and Rott, 1981). Bound antibodies was localized after 1 h-incubation at room temperature with HRPO-conjugated, affinity-purified goat anti-bovine IgG (H+L) (Jackson Immunoresearch Inc.) diluted 1:5,000. All dilutions were made with 10% nonfat-dry milk in NET buffer. Antibody bound conjugate was detected using ECL Western Blot Detection System (Amersham Life Science Inc., Arlington Heights, Il.). Finally, the blot was exposed to Hyperfilm™ (Amersham Life Science Inc.).

4.3 Results

4.3.1 Isolation of RBCV and Respiratory Tract Disease in Response Groups

The results of RBCV isolation and signs of respiratory tract disease were correlated in Table 4.1. Respiratory bovine coronaviruses were isolated from nasal samples of 72 cattle which had mucopurulent nasal discharges, depression and rectal temperatures of 40°C or above (response group 1). Fifty-six of them nasally shed RBCV on day 0 (subgroup 1a, 1b). Sixteen additional cattle became infected during transport (subgroup 1c) while 12 cattle discontinued virus shedding on day 7 (subgroup 1a). Four calves continued to shed RBCV on days 14 and 21. Response group 2 contained 5 cattle which did not show adverse respiratory signs, but secreted RBCV in nasal discharges on day 0 or day 7, and 1 of them continued shedding through day 14. Ten cattle (response group 3) developed severe pneumonia and died from respiratory failure on days 7 to 11.

Virus isolations on nasal swab samples proved that 6 of them shed RBCV on days 0 and 7 while 3 had become infected by day 7. The pneumonic lung tissues of these 9 cattle contained RBCV infectivity reaching titers of 5.0×10^6 plaque forming units per gram. The remaining case was RBCV isolation-negative. However, RBCV-specific genomic portions were detected in the lungs by an RT-PCR assay (Chouljenko et al., 1998a). Eighteen of the 95 remaining cattle did not yield RBCV from sequential nasal swab samples, 11 of them (response group 4) had mild respiratory signs, while the other 7 calves remained clinically healthy throughout the entire epizootic (response group 5).

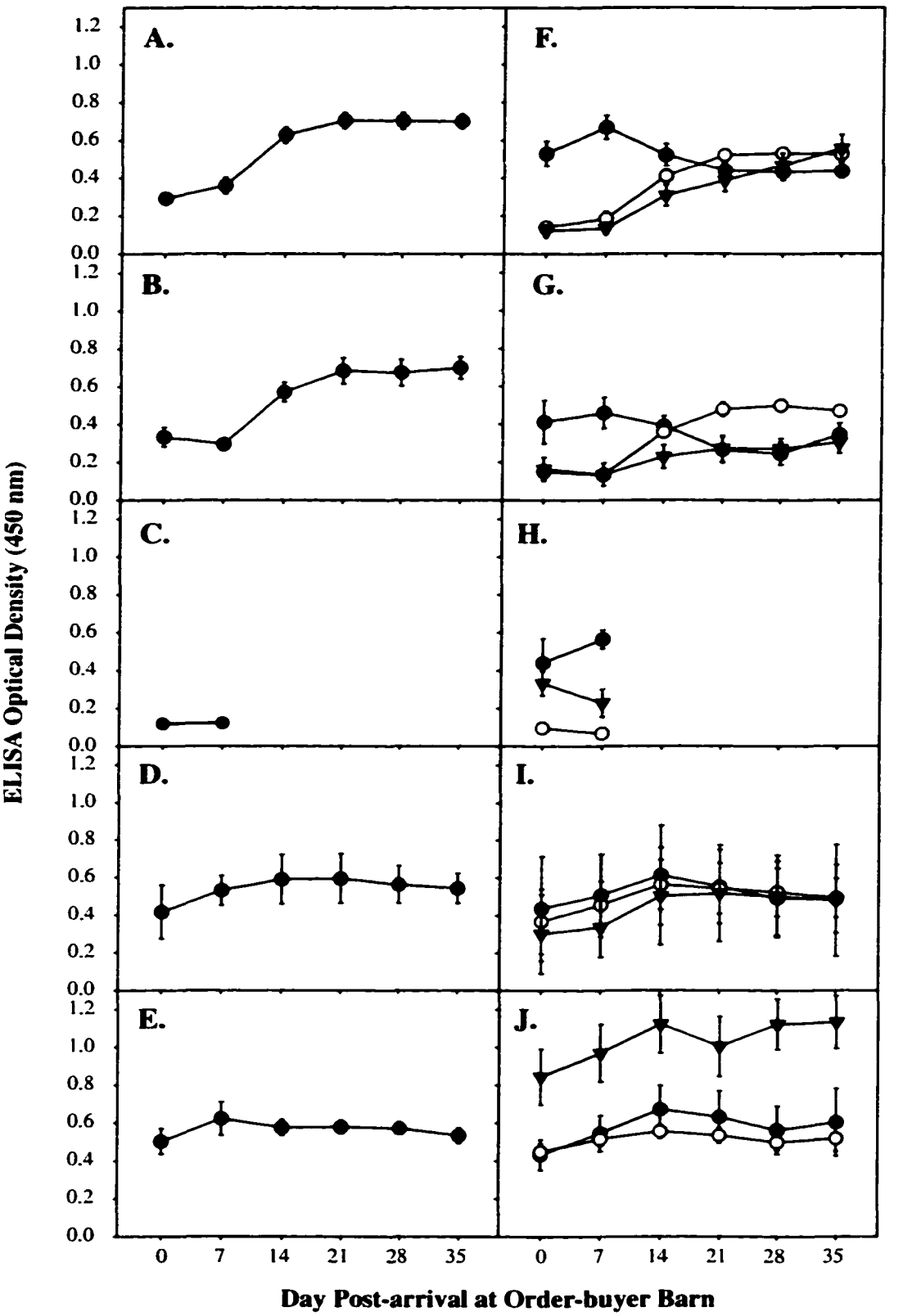
4.3.2 Total and Isotype-specific Antibody Responses to RBCV Infections

Differences in the 5-week total and isotype antibody responses between subgroups 1a, 1b and 1c were not statistically significant, and findings on these 21 cattle were combined for pertinent analyses. Overall kinetics of total and isotype antibody responses for response groups 1 and 2 did not show significant changes (Fig. 4.1A, 4.1B, 4.1F and 4.1G).

Levels of total antibodies to RBCV for all surviving cattle with active RBCV infections of the respiratory tracts (response groups 1 and 2) were initially low with OD_{450} values of 0.29 ± 0.03 and 0.33 ± 0.05 for cattle with and without signs of respiratory distress (Fig. 4.1A and 4.1B). The increases in these levels were statistically significant between days 7 and 14, and then remained at high OD_{450} values reaching

FIG. 4.1: Levels of total (A, B, C, D, E) and isotype antibodies (F, G, H, I, J) to respiratory bovine coronavirus in sera from cattle of response groups 1 (A, F), 2 (B, G), 3 (C, H), 4 (D, I) and 5 (E, J) during the 1997 shipping fever epizootic.

●, total Ig and IgM; ○, IgG1; ▼, IgG2. Data are means ± standard errors of the means (n = 21, 5, 9, 7 and 7 for A+F, B+G, C+H, D+I, and E+J, respectively).



0.71 ± 0.04 and 0.68 ± 0.07. In comparison with those for response group 2, OD_{450} values of IgM isotype for response group 1 were higher on days 0 and 7; and these differences were statistically significant (Fig. 4.1F and 4.1G). As the IgM levels began to decline after day 7, levels of IgG1 and IgG2 isotype antibodies began to rise. Interestingly, calves of response group 1 had a more dramatic increase in IgG2 antibody level than cattle of response group 2 during days 14 and 35.

Nine RBCV isolation-positive cattle with fatal pneumonia in response group 3 had low immune responses on day 0 as OD_{450} values for total Ig, IgM, IgG1, and IgG2 antibodies were 0.12 ± 0.02, 0.44 ± 0.13, 0.10 ± 0.01, and 0.33 ± 0.06, respectively (Fig. 4.1C and 4.1H). Increases in antibody levels were not detected during the 7- or 8-day course of respiratory tract disease pathogenesis except for a minimal initial IgM response.

Cattle in response group 4 started at a relatively high and stable level of total antibody with OD_{450} values of 0.42 ± 0.06, and 0.53 ± 0.03 on days 0 and 7 which showed statistically significant increases from response groups 1 and 2 (Fig. 4.1A, 4.1B and 4.1D). The total antibody level increased to 0.59 ± 0.05 on day 14 and were maintained throughout the testing period. Kinetics of isotype antibody responses reflected that of the total antibody response (Fig. 4.1I). Compared with cattle of response groups 1 and 2, response group 4 cattle also had a higher IgG1 level during the

first 2 weeks, and the increases were statistically significant (Fig. 4.1F, 4.1G and 4.1I). The IgG2 level of response group 4 was significantly higher than that of response group 1 during the first week, and was also substantially higher than that of response group 2 in weeks 2 through 5.

Significant differences were not observed in the total, IgM and IgG1 antibody responses between response groups 4 and 5 during the entire period of this epizootic (Fig. 4.1D, 4.1E, 4.1I and 4.1J). However, during the time of observation, cattle in response group 5 had the highest level of IgG2 isotype antibodies to RBCV of any response group (Fig. 4.1F, 4.1G, 4.1H, 4.1I and 4.1J). The level of IgG2 for these cattle started with an OD_{450} value of 0.84 ± 0.15 on day 0, increased during the following 2 weeks to OD_{450} value of 1.12 ± 0.15 on day 14, and was then maintained.

4.3.3 Antigenicity of RBCV Structural Proteins

Antibody responses to specific viral structural proteins in immunoblotting assays revealed similar reactions among the cattle of each response group, and representative results from a single calf in each response group are presented. Figure 4.2 illustrates the immunoblotting reactions for calf 97TXSF-105 of response group 1 which shed RBCV in nasal secretions on day 7. Antibodies directed against viral structural proteins were not detectable in the serum samples collected on day 0 and day 7, but antibodies of rising levels reacting with HE and S were detected on day 14. Calf 97TXSF-3 of

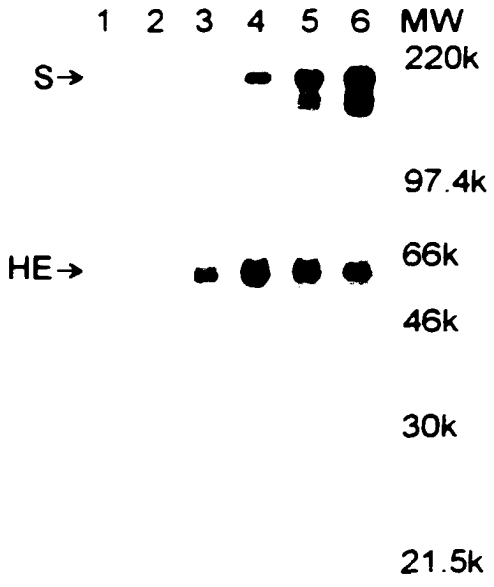


FIG. 4.2: Representative western blot analysis of sera from calf 97TXSF-105 of response group 1.

Lanes 1 through 6 were probed with serum samples collected on day 0, 7, 14, 21, 28 and 35 post-arrival at OBB, respectively.

response group 2 shed RBCV in respiratory secretions on day 7 without adverse clinical signs. The HE- and S-specific antibodies were detected on day 14 (Fig. 4.3). Sera from RBCV isolation-positive and fatal cases of response group 3 failed to react with any of the RBCV structural proteins (data not shown).

Western blot analysis of serum samples collected from RBCV isolation-negative calf 97TXSF-88 of response group 4 which had transient signs of respiratory distress was presented in Fig. 4.4. Strong antibody bindings of HE, S, and N viral proteins were detected on day 7 as cattle arrived in the feedyard, and were maintained for the following 4 weeks. Antibodies directed toward M glycoprotein were detectable at low level on day 14 and 21, waned on day 28, and disappeared on day 35.

Figure 4.5 presents immunoblot reactivities of sera from calf 97TXSF-4 of response group 5 that remained clinically healthy and RBCV isolation-negative throughout the epizootic. The sera contained high levels of specific antibodies to HE, S and N viral proteins on day 0. The HE- or N-specific antibodies in the subsequent serum samples began to decline 3 weeks later, while the S antibody level was maintained or even increased during the next 5 weeks. The M glycoprotein-specific band became visible on day 7, and disappeared 2 weeks later.

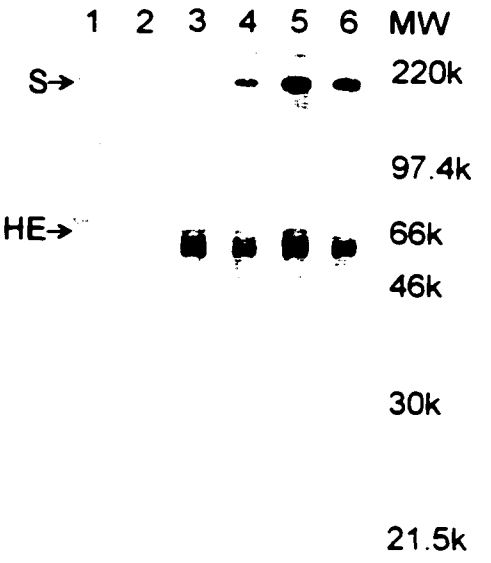


FIG. 4.3: Representative western blot analysis of sera from calf 97TXSF-3 of response group 2.

Lanes 1 through 6 were probed with serum samples collected on day 0, 7, 14, 21, 28 and 35 post-arrival at OBB, respectively.

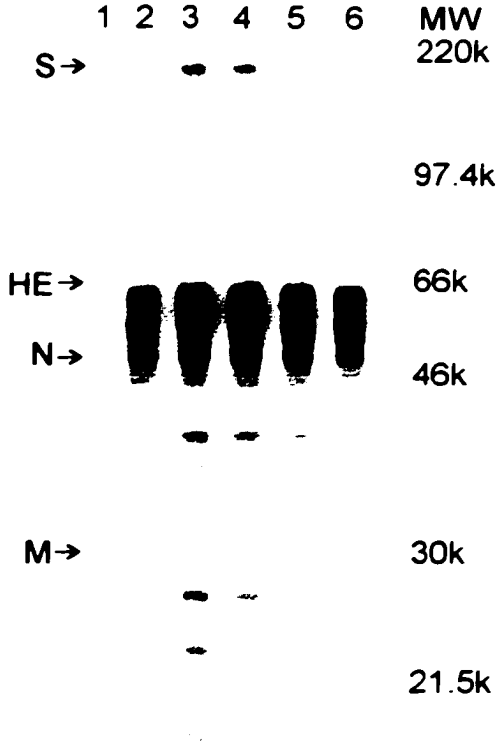


FIG. 4.4: Representative western blot analysis of sera from calf 97TXSF-88 of response group 4.

Lanes 1 through 6 were probed with serum samples collected on day 0, 7, 14, 21, 28 and 35 post-arrival at OBB, respectively.

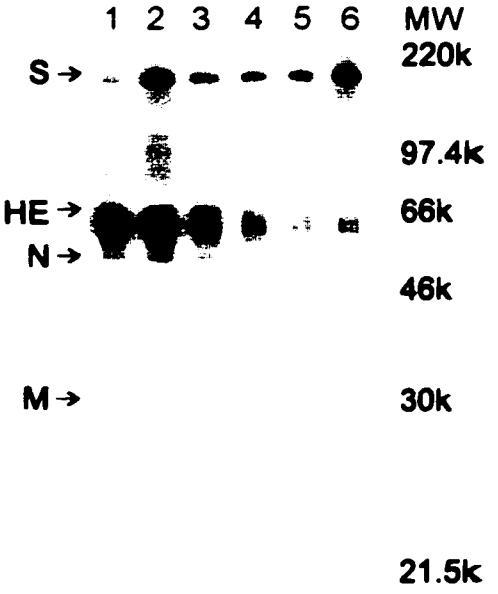


FIG. 4.5: Representative western blot analysis of sera from calf 97TXSF-4 of response group 5.

Lanes 1 through 6 were probed with serum samples collected on day 0, 7, 14, 21, 28 and 35 post-arrival at OBB, respectively.

4.4 Discussion

Isolation results for RBCV and overt signs of respiratory tract disease divided the cattle of this experimentally assessed shipping fever epizootic into 5 response groups. The majority of cattle (response groups 1 and 2) nasally shed RBCV in the early stage of the epizootic. Their initial total antibody levels were low and rapidly increased during the first 2 weeks through brisk responses to HE and S antigens. Increase in IgM appeared first, and was followed by rises in IgG1 and IgG2 as is characteristic for primary immune responses to antigens. With the appearance of HE- and S-specific antibodies, nasal RBCV shedding of most cattle was ceased. This relationship between the RBCV infections and antibody responses can be explained by previous findings in EBCV and human respiratory coronavirus infections which revealed that S and HE elicited virus-neutralizing antibodies (Deregt et al., 1989; Schmidt and Kenny, 1982; Schultze and Herrler, 1992). These findings confirmed a previous report (Heckert et al., 1991) with the difference that antibody responses to N protein could not be detected during the primary immune responses to RBCV infection of these immunologically mature cattle. Based on this previous report (Heckert et al., 1991), our virus isolation results and the detected antibody levels, we concluded that most of these cattle became naturally infected with RBCV shortly before they were assembled at the OBB, and that virus spread was enhanced by stressful conditions during transport and associated crowding.

During the primary immune responses to RBCV, a significantly higher IgM level in the first week and a substantially higher IgG2 level for the last 3 weeks were observed in clinically sick cattle (response group 1) as compared to cattle without adverse respiratory signs (response group 2). Seroconversion was observed in calves between the 14th and the 21st days after intranasal vaccination with modified-live virus of infectious bovine rhinotracheitis (IBR) (LeJan and Asso, 1980). The neutralizing activity in the serum was low at the 14th day, but increased by day 21, and persisted for 2 or 3 months. Other investigators studied primary immune responses of seronegative cattle to IBR after intramuscular or nasal inoculations (Potgieter, 1975; Rossi and Kiesel, 1976). They demonstrated an early, transient and complement-dependent IgM antibody response followed by a complement-requiring IgG antibody response. Bovine complement component 3 (C3) activation was reported to be enhanced by bovine respiratory syncytial virus (BRSV)-specific IgM and IgG1, but not by IgG2 (Kimman et al., 1989a) . In the study of the pathogenesis of BRSV-associated disease, C3 was demonstrated in BRSV-infected parts of the lungs of dead calves, and was suggested to play a role in causing severe dyspnoea (Kimman et al., 1989b). We proposed that an early primary antibody response to RBCV infections included high levels of complement–binding but non-neutralizing antibodies on RBCV-infected cells, which consequently enhanced complement activation and binding as a possible pathologic mechanism of early disease enhancement. Besides its contribution to recovery from the

respiratory tract disease by inducing lysis of RBCV and RBCV-infected cells, complement activation might also play a causative role in adverse respiratory signs by inducing inflammation. Activated complement can increase vessel permeability and smooth muscle contractibility, and activate neutrophils and mast cells. Additionally, massive destruction of RBCV-infected cells could cause severe tissue damage and aggravate respiratory tract disease.

Investigation of fatal cases (response group 3) revealed that the calves were immunologically naïve to RBCV antigens. Antibody responses to any of the major viral structural proteins could not be detected with a sensitive immunoblotting assay. Lack of protective immunity or inability to develop HE- and S-specific virus-neutralizing antibodies against RBCV could have resulted in acute infections of lungs with fatal outcomes in synergy with *Pasteurella haemolytica* infections, typical for shipping fever pneumonia (Hoerlein, 1980; Yates, 1982).

Cattle with respiratory disease signs but not shedding RBCV (response group 4) had relatively high levels of total antibody against RBCV on day 0. Strong RBCV-specific antibody responses against HE, S and N viral proteins had been mounted by the time of their arrival in the feedyard. These findings mirrored the late phase of a primary antibody response to the RBCV infections. Failure in RBCV isolation attempts probably was due to sampling during declining virus excretion. Additionally, RBCV infectivity in nasal secretions might have been neutralized by secretory antibodies through

formation of antibody-virus complexes. Secretory IgA against RBCV was detected in nasal secretions of these cattle (data not shown). These cattle may have played an important role in introducing the RBCV into the large group of susceptible cattle in this severe epizootic.

During the 5-week investigation, 7 cattle remained healthy and RBCV isolation-negative (response group 5). These cattle had the highest level of IgG2 antibody against RBCV for the entire period of the epizootic, and antigens recognized were HE, S and N viral proteins. The S-specific antibody was more stable and persistent than the HE- and N-specific antibodies during the later course of the epizootic. However, antibody reactions with M protein were sporadically detected throughout the sampling period. The serological response patterns of these RBCV-resistant cattle characterized a secondary antibody response to RBCV, the type of protective immunity induced by recovery from initial infection or by effective vaccination and subsequent challenge exposure. Consequently, it was inferred that these cattle had been infected with RBCV, and had recovered from the infections before they were assembled at the OBB. Solid immunity from the initial infection prevented RBCV reinfection throughout this epizootic. This result agreed with previous studies on colostrum-deprived newborn calves which had been experimentally-infected with EBCV and subsequently challenge exposed (Heckert et al., 1991).

A major difference observed in immune responses between RBCV isolation-negative cattle with or without signs of respiratory tract diseases was the ratio of IgG2 to IgG1 which was higher for clinically normal cattle than sick ones. The bovine serum IgG1 has a shorter half-life than IgG2 (Nansen, 1970). Studies on the phagocytosis of *Staphylococcus aureus* by bovine polymorphonuclear neutrophils (PMN) indicated that IgG2 was opsonic for bovine PMN, while IgG1 was not opsonic, and even inhibited IgG2 opsonization (Guidry et al., 1993). In contrast to IgG2, IgG1 caused neither adherence nor phagocytosis *in vitro* by freshly isolated bovine neutrophils and monocytes (McGuire and Musoke, 1980). Competitive inhibition tests indicated that binding of IgG2 exceeded that of IgG1, although IgG1 and IgG2 shared a common Fc receptor on bovine PMN (McGuire et al., 1979; Worku et al., 1994). Re-exposure of cattle to IBR virus resulted in a booster effect on serum antibodies including a transient IgM response as well as a further, rapid increase in IgG (Potgieter, 1975; Rossi and Kiesel, 1976). However, secondary antibody responses seemed to be less responsive to complement enhancement of the neutralizing activity. We hypothesized that during the secondary antibody response to RBCV, free RBCV and RBCV-infected cells were opsonized by high titers of opsonic and RBCV-neutralizing IgG2, leading to enhanced phagocytosis, more efficient elimination of RBCV infectivity, and thus alleviation of clinical signs of respiratory tract disease.

Interestingly, bovine PMN FcR for IgG1 and IgG2 were interferon-γ (INF-γ) inducible (Worku et al., 1994). Bovine IFN-γ enhances IgG2 production by directly affecting suitably stimulated B cells (Estes et al., 1994). The effect of IFN-γ on bovine IgG2 is augmented by interleukin 2 (IL-2). IgM production is suppressed by the presence of IFN-γ alone but this effect is antagonized by IL-2. Bovine IL-4 upregulates the production of IgG1, IgE and IgM, and expression of MHC class II, the low-affinity FcR for IgE (CD23), IL-2R, and surface IgM on bovine B cells (Estes et al., 1995). Future studies on cytokines and related factors should be defined for the humoral as well as cell-mediated immune responses of cattle to RBCV infection.

Remarkable differences were observed in the antibody responses to the major structural proteins of RBCV. The M protein was much less immunogenic than S, HE, and N viral proteins. This observation can be explained by the low molecular mass, structural conformation and inaccessible location of M within the viral envelope. Both HE and S were the viral antigens recognized during the initial stages of the bovine immune response to RBCV infection. The HE glycoprotein induced antibodies earlier than the S glycoprotein. The HE glycoprotein consists of 2 identical, disulfide-linked glycosylated subunits with an N-terminal signal region and a C-terminal anchorage region (Lai, 1990). The S glycoprotein contains an N-terminal signal sequence, a coil-to-coil structure, and a C-terminal hydrophobic membrane-anchoring domain, and

exists as a more stable tetramer. The structure and abundance of the HE protein might favor exposure of epitopes with earlier induction of antibodies. The antibody response to N protein was pronounced during the later phase of the infection when RBCV shedding could not be detected by virus isolation attempts, but a modified polymerase chain reaction detected RBCV genomic regions in lung samples (Chouljenko et al., 1998a). We hypothesized that the relatively late appearance of N-specific antibodies might depend on the quantity of N in the virions, its structural binding to the RNA genome, and its internal site within the viral envelope. Findings on cell-mediated immunity to mouse hepatitis virus revealed that CD8-bearing lymphocytes influenced recovery from infection with N protein as the major target for cytotoxic lymphocytes (Stohlman et al., 1993). Therefore, HE, S and N proteins should be the major viral antigens to be included in future vaccines for cattle to achieve efficient prevention of RBCV as well as EBCV infections.

CHAPTER 5

INFECTION OF POLARIZED EPITHELIAL CELLS WITH RESPIRATORY AND ENTERIC TRACT BOVINE CORONAVIRUSES AND RELEASE OF VIRUS PROGENY*

5.1 Introduction

Bovine Coronavirus (BCV) infections induce severe diarrhea in neonatal calves and are referred to as enteropathogenic BCV (EBCV). They may also cause winter dysentery in adult dairy cattle (Mebus et al., 1973; Saif et al., 1988). Furthermore, the high rate of BCV isolations from nasal samples of feedlot cattle with signs of respiratory tract infections implies an etiological role in respiratory diseases (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). These isolates are considered respiratory BCV (RBCV). The BCV are spherical and enveloped, have a diameter of about 80 nm, and possess a single-stranded nonsegmented RNA genome with positive polarity (Spaan et al., 1988). Virions contain 5 major structural proteins: the nucleocapsid protein (N), the transmembrane protein (M), the hemagglutinin/esterase protein (HE), the spike protein (S) and the envelope protein (Brown and Brierly, 1995; Cavanagh et al., 1990; Lai, 1990).

* Reprint for letter of permission by the American Journal of Veterinary Research is on page 214.

Observations of the interaction of wild-type EBCV strains with cultured cells were limited, because they could not be adapted to a variety of bovine cells with the exception of the Mebus strain (Mebus et al., 1973; Storz et al., 1981). However, isolation and propagation of the wild-type EBCV was successful in the human rectal tumor cell-18 line (HRT-18) which was established from an adenocarcinoma of the lower gastrointestinal tract by Tompkins and coworkers (Laporte et al., 1979; St. Cyr-Coats and Storz, 1988; Tompkins et al., 1974). Monolayers of these polarized epithelioid cells exhibited several structural and functional features of epithelial cells that are targeted by BCV in natural infections (Doughri and Storz, 1977). The apical and lateral surfaces of the HRT-18 cells are modified by abundant filiform microvilli (Payne and Storz, 1990), and the lateral membranes possess a pronounced system of intercellular adhesions (Williams, 1983).

Research on the interaction of influenza viruses with epithelial cells of the chorioallantois of chicken embryos indicated that the release of progeny virus occurred exclusively by budding at the apical cell surface (Murphy and Bang, 1952). Release of the virus thus was directional, occurring at only one side of this epithelial cell layer. More recently, it was reported that the directional budding of several enveloped viruses occurred from either the apical or the basolateral surfaces of epithelial cells in culture (Jones et al., 1985; Rodriguez-Boulan and Sabatini, 1978; Roth et al., 1983). The major aim of this investigation was to assess the interaction of enteric and respiratory strains

of BCV with the polarized epithelioid G clone of HRT-18 cells following directional exposure. Cytopathic changes induced under these polarizing cytological conditions, as well as the release of hemagglutinins (HA) and viral infectivities from different cellular domains were assessed.

5.2 Materials and Methods

5.2.1 Cells, Media and Polarizing Cultural Conditions

The G clone of HRT-18 cells was used at the 18th passage level for these investigations. Monolayers of G clone cells were grown on polyester membranes with a pore size of 400 nm. These membranes had been treated to facilitate culturing of cells, and were inserted into 6-well cluster plates (Costar, Cambridge, Mass.). Cells were cultured in DMEM with 44 mM NaHCO3 buffer and penicillin (100 units/ml) - streptomycin (100 µg/ml). Medium supplemented with 5% fetal calf serum was used for cell propagation, and serum-free medium was used during virus propagation. Confluent monolayers of G clone cells on the inserts of the wells of the plates were washed 2 times with serum-free Dulbecco's PBS (calcium and magnesium free) and exposed to 1.4 ml virus preparation from apical or 2.8 ml virus preparation from basolateral surfaces. Volumes of 1.4 ml and 2.8 ml of the DMEM were added to the spaces of the opposite, uninoculated surfaces to balance the volumes. The volumes were recommended by the manufacturers of the inserts. Uninfected cells were used as negative control, while cells infected with vesicular stomatitis virus (VSV) were used as

positive control. Following an absorption period of 1 h at 37°C, excess inoculum was removed, and cells were covered with fresh DMEM. Infected cluster plates were incubated at 37°C and cytopathic changes were monitored by microscopic examination.

5.2.2 Processing for Scanning Electron Microscopy (EM)

Parallel confluent control and inoculated monolayers of G clone cells on inserts in the wells of plates were treated with EM stock fixative (1.25% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer pH 7.4), washed in 0.1 M sodium cacodylate buffer with 5% sucrose at pH 7.4. Segments of about 5 mm^2 are cut from the monolayers on the membranes with razor blades, and incubated in sodium cacodylate buffered solutions (pH 7) of 1% osmium tetroxide for 30 min, 1% tannic acid for 15 min and again in 1% osmium tetroxide for 15 min, rinse in distilled water, dehydrated in a gradient alcohol series, critical point dried, mounted with carbon tabs, and sputter coated with gold-palladium. The samples were viewed with a Cambridge S-150 scanning electron microscope.

5.2.3 Giemsa Staining

Parallel confluent monolayers of G clone cells on the membranes was fixed in Bouin's fixative, stained in 1:10 dilution of Giemsa reagents in 1× Giemsa buffer (A.J.P. Scientific Inc., Clifton, N.J.), and mounted with CoverBond™ mounting media (Division of American Hospital Supply Co., Genearl Office, McGAW PARK, Il.).

5.2.4 Virus Strains

The 2 EBCV strains, L9 (cell-culture adapted strain) and LY138 (wild-type strain), used in these investigations had been passaged in cell cultures 80 and 2 times, respectively. Two additional wild-type strains of RBCV, LSU-94LSS-051 and OK-0514, were used at their 2nd and 3rd G clone cell passages, respectively. The viral infectious titers were 1.0 to 4.0 $\times$ 10^8 plaque forming units per ml (PFU/ml). The VSV propagated in chicken embryos and once in G clone cells, in which it had a titer of 2.0 $\times$ 10^5 PFU/ml, was used as a comparative virus control.

5.2.5 Directional Sampling of Virus Released from BCV-infected G Clone Cells

Fluid samples (200 µl) were taken at different intervals (0 h, 6 h, 12 h, 24 h) from both the top and the bottom of the infected G clone cell monolayers growing on inserts in cluster plates. After each sampling, the same volume (200 µl) of fresh DMEM was added to restore the original volume, so that the last samplings had the original volume of fluid in the inserts after a 30-h incubation. The whole fluid was removed as the last samples collected from the bottom wells of the infected monolayers 30 h after inoculation, while the fluid in the inserts was frozen with the monolayers at –70°C and thawed at room temperature. Then, the cells were scrapped off and placed with the media into sterile Eppendorf tubes. The cell suspensions were then centrifuged at 4°C with a speed of 1,500 $\times$ g for 20 min. Supernatant fluids were transferred to Eppendorf

tubes and identified as the 30-h cell free samples collected from the apical surfaces of the infected monolayers.

5.2.6 Harvest of BCV-infected G Clone Cell Monolayers

An amount of 1 ml of DMEM was added to the cell sediment, and the cell suspensions were treated with sound 2 times for 15 s at a power setting of 2 (Branson Sonifier Cell Disruptor 200, Branson Ultrasonics Co., Danbury, Conn.) to release intracellular virus.

5.2.7 Assessment of Pore Size of Inserts for Virus Passage

An exploratory experiment assessing the ability of RBCV to penetrate the 400 nm membrane pores was done as follows: virus preparations at volumes of 1.4 ml were placed into the polyester membrane inserts and 2.8 ml were placed into the bottom wells. Corresponding volumes of DMEM were added to the opposite sides to balance the volume. Plates were incubated at 4°C for 1 h. The fluids in the wells and on the membrane inserts were then titered for HA activities.

5.2.8 Assays for HA

The assay for HA was performed as reported (Storz et al., 1999b and c) with washed rat red blood cells prepared to 0.5% with PBS pH7.4, containing 0.05% bovine serum albumin.

5.2.9 Plaque Assays

The plaque assay was performed according to recently published procedure (Lin et al., 1997b), using 6-well flat-bottom tissue culture plates with confluent monolayers of G clone cells.

5.3 Results

5.3.1 Observations on Surfaces and Intercellular Binding of Control G Clone Cell Monolayers

The G clone cell monolayers on the porous membranes were examined by scanning EM and light microscopy, which revealed high cell density and complete tight intercellular contact as unique characteristics. Figure 5.1 depicts the surface of the G clone cell monolayers with abundant microvilli and tight intercellular binding.

5.3.2 Cytopathic Changes Induced in G Clone Cells Following Directional Infection with RBCV and EBCV

Cytopathic changes consisting of cell fusion were detected at 12 h after apical inoculation. These changes were abundant by 24 h while basolaterally inoculated cultures had only minimal changes (Table 5.1). Infected cells formed polykaryons in all cultures at 30 h after inoculation, irrespective of the route of inoculation. A difference between cells inoculated with respiratory or enteric BCV isolates was not observed. Cells inoculated with VSV as virus controls developed cytocidal changes at 12 h after inoculation by either route, and complete cell detachment and lysis had occurred by

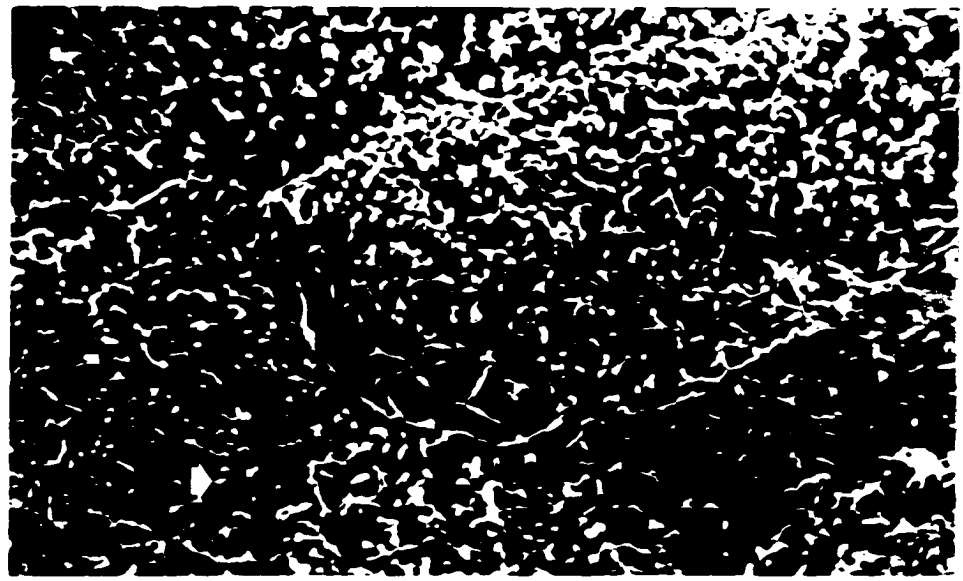


FIG. 5.1: Scanning electron micrograph of a monolayer of uninfected G clone cells.

The surface is illustrated with microvilli (white arrow), and tight intercellular arrangement (black arrow) (× 5700); bar = 4μm.

Table 5.1: Cytopathic Changes in G Clone Cells Following Apical and Basolateral Exposure to Respiratory and Enteropathogenic Bovine Coronaviruses and Vesicular Stomatitis Virus

Strains of BCV	Exposure Sites	Hours After Inoculation				
		0	6	12	24	30
RBCV-LSU-94LSS-051-2	A	-	-	±	++++	++++
	B	-	-	-	±	++++
RBCV-OK-0514-3	A	-	-	±	++++	++++
	B	-	-	-	±	++++
EBCV-L9-80	A	-	-	±	++++	++++
	B	-	-	-	±	++++
EBCV-LY138-2	A	-	-	±	++++	++++
	B	-	-	-	±	++++
VSV*	A	-	-	±	++++	++++
	B	-	-	±	++++	++++

BCV: bovine coronavirus; RBCV: respiratory bovine coronavirus; EBCV: enteropathogenic bovine coronavirus; VSV: vesicular stomatitis virus; A: apical; B: basolateral, -: no cytopathic changes; ±: beginning cytopathic changes, consisting of cell fusion ≤ 10% of the monolayers involved; ++++: complete fusion of over 90% of cells in monolayers; *: VSV induced rounding and rapid lysis of the G clone cells.

30 h. Uninoculated control cells maintained a dense confluent monolayer of small cuboidal epithelioid cells on the polyester membrane inserts.

5.3.3 Hemagglutinin and Infectious Virus Release from G Clone Cells Directionally Inoculated with RBCV and EBCV

Hemagglutinins interacting with rat RBC were released into the apical medium irrespective of apical or basolateral exposure with increasing titers reaching levels of 32 to 64 by 30 h (Table 5.2). The basolateral samples did not carry detectable HA for 30 h. Experiments with directional placement of virus inocula permitted passage of BCV isolates through the membranous inserts with a pore size of 400 nm within 1 h (data not shown). The highest infectivity titers of 5.2×10^7 PFU/ml in the 12-h samples and 4.2×10^8 PFU/ml in the 24-h samples were present in the apical medium of G clone cells apically or basolaterally exposed with either RBCV or EBCV (Table 5.3). Samples collected from the basolateral medium of cells exposed by apical or basolateral routes had minimal infectivity titers that were 4 or 5 logs lower than those of the apical samples. The G clone cells inoculated with VSV released virus through apical and basolateral surfaces with equal titers when exposed by either route (Table 5.3).

5.3.4 Hemagglutinins and Viral Infectivities in Homogenized G Clone Cells Directionally Inoculated with RBCV and EBCV

Cell cultures that were homogenized at 30 h after the different modes of directional inoculation had HA titers of 16 to 128 and viral infectivity titers of 1.2×10^6

Table 5.2: Hemagglutinin Titers Released by G Clone Cells Following Directional Exposure to 4 Strains of Respiratory and Enteropathogenic Bovine Coronaviruses

Strains of BCV	Sites of		HA Titers X Hours After Inoculation				
	Exposure	Sampling	0	6	12	24	30
RBCV-LSU-	A	A	<2	<2	16*	32*	64
94LSS-051-2	A	B	<2	<2	<2*	<2*	<2
	B	A	<2	<2	<2*	16*	32
	B	B	<2	<2	<2*	<2*	<2
RBCV-OK-0514	A	A	<2	<2	16*	32*	32
-3	A	B	<2	<2	<2*	<2*	<2
	B	A	<2	<2	<2*	32*	32
	B	B	<2	<2	<2*	<2*	<2
EBCV-L9-80	A	A	<2	<2	8*	32*	32
	A	B	<2	<2	<2*	<2*	<2
	B	A	<2	<2	<2*	16*	64
	B	B	<2	<2	<2*	<2*	<2
EBCV-LY138-2	A	A	<2	<2	8*	64*	32
	A	B	<2	<2	<2*	<2*	<2
	B	A	<2	<2	<2*	32*	64
	B	B	<2	<2	<2*	<2*	<2

*: samples were assayed for plaque forming infectivity in table 4.3; Other notes as table 5.1. Hemagglutinin (HA) titers were expressed as hemagglutination units (HAU)/50 µl, reflecting the highest titer with complete agglutination.

Table 5.3: Titers of Hemagglutinins and Viral Infectivities of Virus after Directional Inoculation of G Clone Cells with Respiratory and Enteropathogenic Bovine Coronaviruses and Vesicular Stomatitis Virus

Strains of BCV	Sites of		12 Hours PI		24 Hours PI	
	Exposure	Sampling	HAU	PFU/ml	HAU	PFU/ml
RBCV-LSU	A	A	16	5.2×10^7	32	4.2×10^8
-94LSS-051-2	A	B	<2	2.8×10^2	<2	6.4×10^3
	B	A	<2	7.0×10^3	16	6.8×10^7
	B	B	<2	1.3×10^4	<2	1.7×10^4
RBCV-OK-0514	A	A	16	4.8×10^7	32	2.4×10^8
-3	A	B	<2	2.0×10^2	<2	9.6×10^3
	B	A	<2	8.0×10^3	32	2.4×10^8
	B	B	<2	1.0×10^4	<2	2.4×10^4
EBCV-L9-80	A	A	8	4.0×10^7	32	1.6×10^8
	A	B	<2	4.0×10^1	<2	4.0×10^3
	B	A	<2	1.4×10^3	16	2.0×10^7
	B	B	<2	3.6×10^4	<2	9.6×10^3
EBCV-LY138-2	A	A	8	8.0×10^6	64	6.8×10^7
	A	B	<2	$<4.0 \times 10^1$	<2	9.6×10^2
	B	A	<2	8.0×10^3	32	2.6×10^7
	B	B	<2	1.8×10^4	<2	4.8×10^3
VSV	A	A	<2	2.0×10^2	<2	1.0×10^5
	A	B	<2	$<4.0 \times 10^1$	<2	8.0×10^1
	B	A	<2	4.0×10^2	<2	1.4×10^5
	B	B	<2	4.0×10^2	<2	1.2×10^4

PI: postinfection; PFU: plaque forming units; other notes as table 5.2.

to 4.0×10^7 PFU/ml (Table 5.4). No significant difference in these titers was observed when RBCV or EBCV isolates were inoculated by the 2 different routes.

5.4 Discussion

Our results demonstrate that polarized epithelioid G clone cells had maximal susceptibility to BCV infections from apical domains and minimal susceptibility from basolateral surfaces, but infectious progeny BCV virions were asymmetrically released from the apical surfaces of these cells. VSV entered and exited the G clone cells through both apical and basolateral surfaces.

Monolayers of polarized epithelioid G clone cells behave both structurally and functionally like epithelial cells, the type of cells targeted by BCV in natural infections. Monolayers of parent HRT-18 cells are composed of cells oriented with a basally located nucleus and the bulk of the cytoplasm above the nucleus. Intercellular adhesions consisting of desmosomes and maculae adherens are found connecting the cells to each other in culture. Microvilli are present both at the apical surface of the cells exposed directly to the cell culture medium and also in lateral areas between cells not connected by intercellular junctions and adhesions (Williams, 1983). The apical surfaces of the parent HRT-18 cells are modified by abundant small filiform microvilli (Payne and Storz, 1990). The HRT-18 cells exhibit features of polarized epithelial cells characterizing both enterocytes of gastrointestinal tracts and ciliated epithelial cells of respiratory tracts (Tucker and Compans, 1993). Recently, the epithelial cells of the

Table 5.4: Hemagglutinin Titers and Virus Infectivities in Harvest of G Clone Cells

Strains of BCV	Exposure Sites	HAU	PFU/ml
RBCV-LSU-94LSS-051-2	A	16	1.2×10^6
	B	32	9.6×10^6
RBCV-OK-0514-3	A	32	6.8×10^6
	B	128	4.0×10^7
EBCV-L9-80	A	32	3.0×10^7
	B	32	3.0×10^7
EBCV-LY138-2	A	64	4.8×10^6
	B	64	4.8×10^6
VSV	A	<2	2.0×10^4
	B	<2	3.6×10^4

Notes as table 5.3.

respiratory tract of cattle were found to be highly susceptible to RBCV(Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b).

The morphology and morphogenesis of wild-type strain LY138 of EBCV infecting enterocytes of experimentally inoculated calves were first investigated by Doughri and coworkers (Doughri et al., 1976). Uptake of EBCV occurred through fusion of viral envelopes with the plasmalemma of the brush border with abundant virus binding to microvilli, or by entry into intercellular spaces and interaction with the lateral cell membranes of adjacent intestinal epithelial cells.

The abundance and unique distribution of microvilli of G clone cells point to specific mechanism by which BCV infection may be initiated in these cells. Since it is now evident that BCV is not absolutely restricted to binding to the apical plasma membrane domains, it is reasonable to conclude that BCV receptor molecules are expressed on microvilli of both apical surfaces and intercellular areas of G clone cells to mediate virus infection. However, fewer microvilli at lateral intercellular areas makes the G clone cells minimally susceptible to BCV when infected basolaterally. Then, progeny virions released from apical surfaces reinfect the target cells from this domain and cause C.P.E. which was delayed for about 12 h, when compared with apical exposure.

Recently, Schultze and Herrler found that infection of MDCK I cells with strain L9 of EBCV and influenza C virus is restricted to the apical cell surface (Schultze and

Herrler, 1995). Fuller et al found that VSV infects primarily through the basal surface of MDCK cells (Fuller et al., 1984). These findings seem to differ from our results. The different cell types used in the experiments may account for the observed divergence.

The MDCK cell is a well-characterized epithelial cell line and contains all the elements of a polarized cell. The cell exhibits features characteristic of native renal tubular epithelium, including sparse microvilli, junctional complexes and defined lateral spaces (Cereijido et al., 1978; Misfeldt et al., 1976). However, up to now, investigations have not indicated that MDCK cells have microvilli at lateral intercellular areas not connected by the tight junctions. These lateral areas of G clone cells may be accessible to BCV when infected basolaterally. In accordance, EBCV-LY138 had been detected within the intercellular space of adjacent intestinal epithelial cells (Doughri et al., 1976).

Doughri and coworkers found that release of wild-type strain LY138 of EBCV from infected cells in the jejunal and ileal mucosa of calves occurred by lysis and fragmentation of the apical plasmalemma and flow of the cytoplasm with its contents into the gut lumen (Doughri et al., 1976). Release also occurred by digestion and lysis of extruded infected cells or by fusion of virus-containing cytoplasmic vacuoles with the apical plasmalemma and liberation of their contents.

It was reported by Rossen et al (1995) that 2 members of the family *Coronaviridae*, the porcine transmissible gastroenteritis virus (TGEV) and mouse hepatitis virus

(MHV-A59) both enter polarized epithelial cells through the apical membrane. However, the release of newly synthesized TGEV occurred preferentially from the apical domains of LLC-PK1 cells while MHV was released preferentially from the basolateral membrane of mTAL cells. A more recent analysis revealed that the envelope glycoproteins of enveloped viruses accumulated on the same membrane domain from which virus release occurred (Jones et al., 1985; Roth et al., 1983; Stephens and Compans, 1986). The glycoproteins that assemble at the plasma membrane are directionally transported to the surface from which virus buds when their genes are expressed in the absence of other virus-specific polypeptides. A hypothesis predicts that the site of insertion of the envelope glycoproteins determines the site of viral assembly. The glycoproteins of these enveloped viruses are directionally processed in the endoplasmic membranes for BCV to be released at the plasma membrane of the apical surfaces, because BCV is enveloped on endoplasmic membranes and is released preferentially from apical cellular domains. It is still not clear whether the envelope glycoproteins are the only targeted component that defines the domain from which BCV is selectively released.

The G clone cells are structurally and functionally similar to epithelial cells in intestinal and respiratory tracts with specific apical modification of villi and cilia. The specific pathway of BCV release reflects the characteristic intracellular transport and surface expression of viral proteins which provide important information about the

mechanism by which membrane proteins are targeted to specific plasma membrane domains of the epithelial layers. Restricted specific routes of viral entry or release determine the pathogenesis of viral infection as well as the potential mechanisms utilized by virus to circumvent the epithelial barrier. Although BCV can enter target cells only through apical surfaces in the case of epithelial cells with functional tight junctional complexes, under pathogenic conditions when junctional complexes detach, BCV may infect the epithelial cells through both apical and lateral areas.

CHAPTER 6

COMPARISON OF FUSOGENIC PROPERTIES OF RESPIRATORY AND ENTERIC TRACT BOVINE CORONAVIRUSES

6.1 Introduction

Bovine coronaviruses (BCV) were isolated in 1970 as a cause of severe diarrhea in neonatal calves (Mebus et al., 1973), may also be responsible for winter dysentery of adult cattle (Saif et al., 1988). These viruses were referred to as enteropathogenic bovine coronaviruses (EBCV). The Mebus strain of EBCV was isolated by adaptation to a specific primary bovine fetal kidney (BFK) cell line (Mebus et al., 1973). It induced minimal cytopathic changes during multiple passages. Addition of trypsin to the culture medium activated a factor enhancing cytopathic expression and polykaryocytosis of the cell culture-adapted strain L9 (EBCV-L9) in bovine fetal brain (BFB) and thyroid (BFTy) cell cultures (Storz et al., 1981). The spike (S) glycoprotein, one of the 4 envelope-associated structural proteins in EBCV, facilitates virus attachment to susceptible cells and cell fusion (Abraham et al., 1990b; Lai, 1990). Investigation on the polypeptide profile of EBCV-L9 propagated in human rectal tumor-18 (HRT-18) cells and bovine fetal spleen cells (D_2BFS) revealed that trypsin cleaved the 190-kDa S protein into 110-kDa S1 and 100-kDa S2 subunits which was required for cell fusion (St. Cyr-Coats et al., 1988b). The S2 subunit of the EBCV Quebec strain was identified

to mediate cell fusion because S2 recombinant baculovirus induced large syncytia in *Spodoptera frugiperda* (Sf9) cells (Yoo et al., 1991). In contrast to EBCV-L9, wild-type EBCV could not be propagated in cultured bovine cells even in the presence of trypsin. However, wild-type EBCV strains could be isolated in the HRT-18 cell line, which was derived from human rectal adenocarcinoma (Laporte et al., 1979; Tompkins et al., 1974). This process was enhanced by addition of trypsin to the culture medium (St. Cyr-Coats et al., 1988a; Storz et al., 1981).

Antigens of BCV were detected in nasal swab samples from calves with signs of pneumoenteritis (Heckert et al., 1990; Möstl et al., 1988; Saif et al., 1986). However, conventional virus isolation methods did not detect BCV from respiratory tract samples of adult cattle until the unique G clone of HRT-18 cells was used (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). Hundreds of coronaviral strains were isolated in G clone cell cultures from nasal samples of adult cattle experiencing acute respiratory distress and lungs of cases with fatal pneumonia (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). The G clone cell-dependent coronaviruses were identified as respiratory bovine coronaviruses (RBCV). Phenotypic properties of RBCV distinguishing them from EBCV were their ease of isolation in the 1st G clone cell passage and their high fusogenicity at neutral pH ranges without the need for trypsin enhancement (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b).

Virus envelope-cell membrane fusion and virus-induced cell fusion are directly related to the mechanisms of viral entry into host cells (Payne et al., 1990a and b). The extensive and trypsin-independent cell fusion induced by all the RBCV isolates prompted us to analyze this unique polykaryon formation. Two representative RBCV strains were selected from a collection of hundreds of wild-type RBCV isolates with identical properties for comparison with 2 well-defined prototype strains of EBCV. The formation of polykaryons during RBCV and EBCV replications in G clone cells was compared, and the integrity of cytoplasmic membranes and nuclei of the RBCV-induced polykaryons were monitored and related ultrastructural evidence for virus morphogenesis and replication within the polykaryons.

6.2 Materials and Methods

6.2.1 Cells and Virus Preparations

The G clone was established from the parent HRT-18 cells (Lin et al., 1997b; Storz et al., 1996). The G clone cells were used at the 24th passage and grown to confluence in 6-well cluster plates (Becton-Dickinson, Lincoln Park, N.J.) with DMEM containing 44 mM $NaHCO_3$ and supplemented with 5% fetal calf serum. The highly cell culture-adapted strain EBCV-L9 was derived from EBCV-Mebus strain by sequential passages through different bovine and parent HRT-18 cells, and used at its 80th G clone cell passage (St. Cyr-Coats et al., 1988b; Zhang et al., 1991). The virulent wild-type strain EBCV-LY138 from intestinal samples of diarrheic, newborn calves was initially

maintained by oral inoculation of conventional calves and induction of enteritis (Doughri et al., 1976). It was used at its 2nd G clone cell passage. Two wild-type strains of RBCV, LSU-94LSS-051 and OK-0514, were used at their 3rd and 4th G clone cell passages, respectively. Virus stocks were prepared from cells infected at a multiplicity of 0.1 plaque forming units (PFU) per cell, incubated for 4 to 5 days at 37°C in serum-free DMEM, subjected to 3 cycles of freezing-thawing, 2 pulses of sonication on ice for 15 s at a power setting of 4 (Branson Sonifier Cell Disruptor 200, Branson Ultrasonics Co., Danbury, Conn.), and frozen at −70°C. Titers of infectious virus in these preparations ranged from 2.0 to 6.0 × 10^7 PFU/ml.

6.2.2 Analysis of Sequential BCV-induced Polykaryon Formation

Confluent monolayers of G clone cells were washed 2 times with serum-free Dulbecco's PBS (calcium and magnesium free) and inoculated with 0.5-ml virus preparation containing 1.0 to 3.0 × 10^7 PFU. Uninfected cells were used as negative controls. Excess inoculum was removed after absorption for 1 h at 37°C, and cells were covered with 3 ml fresh serum-free DMEM. Inoculated cluster plates were incubated at 37°C, and cytopathic changes were monitored by phase-contrast microscopy (Zeiss, Germany).

6.2.3 Testing of Membranes of RBCV-induced Polykaryons by Trypan Blue Exclusion

Detached polykaryons were collected at 2-h intervals from the medium by centrifugation at 1,500 × g for 20 min, washed with and resuspended in PBS, or treated with methanol. Both types of preparations were stained with 0.4% trypan blue (GIBCOL BRL, Grand Island, N.Y.). Aggregates of uninfected G clone cells were created by detaching them from the monolayer with 0.25% trypsin treatment to serve as normal uninfected cell controls.

6.2.4 Demonstration of Nuclei within RBCV-induced and Detached Polykaryons by Giemsa Staining

Aliquots of floating polykaryons, obtained as described above, were cytospun onto glass slides (Shandon Southern Products Inc. Astmoor, Runcorn, Cheshire) at 1,100 rpm for 5 min and air-dried. The preparations were treated with Bouin's fixative (1.2% aqueous pricric acid, 40% formaldehyde), stained in 1:10 dilution of Giemsa reagents in 1× Giemsa buffer (A.J.P. Scientific Inc. Clifton, N.J.) and mounted with Coverbond™ media (Division of American Hospital Supply Coporation General Offices, McGAW PARK, Il.). Cellular aggregates of uninfected G clone cell culture were obtained as described above and used as negative controls.

6.2.5 Detection of RBCV Antigen in Polykaryons by Indirect Immunofluorescent Assay (IFA)

Floating polykaryons and cells removed from monolayers by trypsin treatment were collected from infected G clone cell cultures, cytospun onto glass slides, and stained by IFA. These specimens were fixed for 30 min with 3% paraformaldehyde in PBS for detecting plasma membrane-associated RBCV antigens. Parallel preparations were further treated for 5 min with acetone at −20°C for tracing cytoplasmic antigens. Anti-S monoclonal antibody (Mab) 43C2 (Hussain et al., 1991) and polyclonal BCV antiserum 1745 (Storz and Rott, 1980; Storz and Rott, 1981) were used at 1:50 dilutions. Fluorescein isothiocyanate (FITC)-labeled affinity purified goat anti-mouse and anti-bovine IgG were diluted at 1:50 (Kirkegaard & Perry Laboratories Inc. Gaithersburg, Md.), and served as indicator antibodies. The antibody-treated cytologic preparations were then counter stained with Evans blue, and examined with a UV microscope (Olympus AH-2, Olympus Optical Co. LTD, Japan).

6.2.6 Processing of RBCV-induced Polykaryons for Ultrastructural Analysis

The procedures described by Payne et al (Payne et al., 1990b) were modified. Briefly, floating polykaryons were washed once with PBS, and fixed at 4°C overnight with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). These preparations were sedimented, incubated for 1 h in a solution of 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer, washed in 0.1 M sodium

cacodylate buffer, and stained *en bloc* for 1 h with 0.2% uranyl acetate in distilled water. The preparations was dehydrated in an ascending alcohol gradient, embedded in Epon-Araldite resins (Mollenhauer, 1964), and polymerized at 60°C. Sections of 60-90 nm thickness were prepared, stained with uranyl magnesium acetate and lead citrate, and examined with a Zeiss EM-10C electron microscope (Leo electron microscopy Inc. Germany).

6.3 Results

6.3.1 Comparison of EBCV and RBCV-induced Fusion in G Clone Cells

The EBCV caused minimal fusion of the G clone cells which was identical in cultures infected with the highly cell culture-adapted avirulent strain L9-80 and the wild-type virulent strain LY138-2 (Fig. 6.1A). In contrast, the 2 RBCV strains, RBCV-OK-0514-3 and RBCV-LSU-94LSS-051-4, induced the earliest cell fusion involving about 10% of the cell monolayers at 12 h postinfection. Polykaryons lost anchorage to culture substrates and detached from the monolayers in synchrony with fusion. Most of the cells in monolayers infected by RBCV were recruited into floating, pluriform and outreaching polykaryons by 24 h postinfection (Fig. 6.1B).

6.3.2 Properties of RBCV-induced Polykaryons

Polykaryons collected at 12 h postinfection with RBCV-OK-0514-3 had plasma membranes excluding trypan blue (Fig. 6.2A). Polykaryons floating in the media progressively lost their membrane integrity after another 4-h incubation at 37°C, when

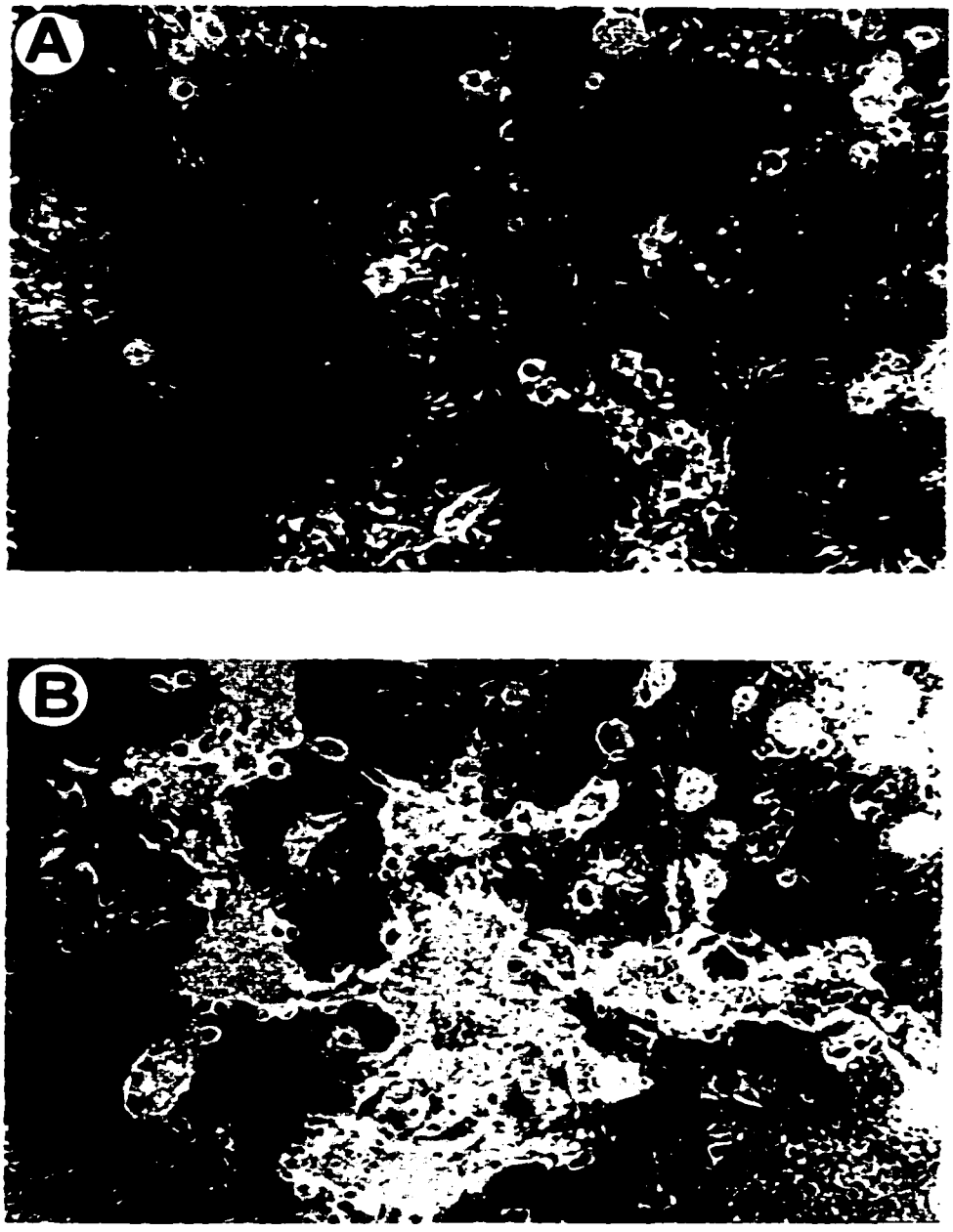


FIG. 6.1: Cytopathic changes in G clone cell cultures infected with EBCV-L9-80 (A) or RBCV-OK-0514-3 (B) at 24 h postinfection.

Limited fusions (solid arrow) in monolayers of EBCV-infected cultures, and large and interconnecting polykaryons (solid arrow) in RBCV-infected cultures (× 66).

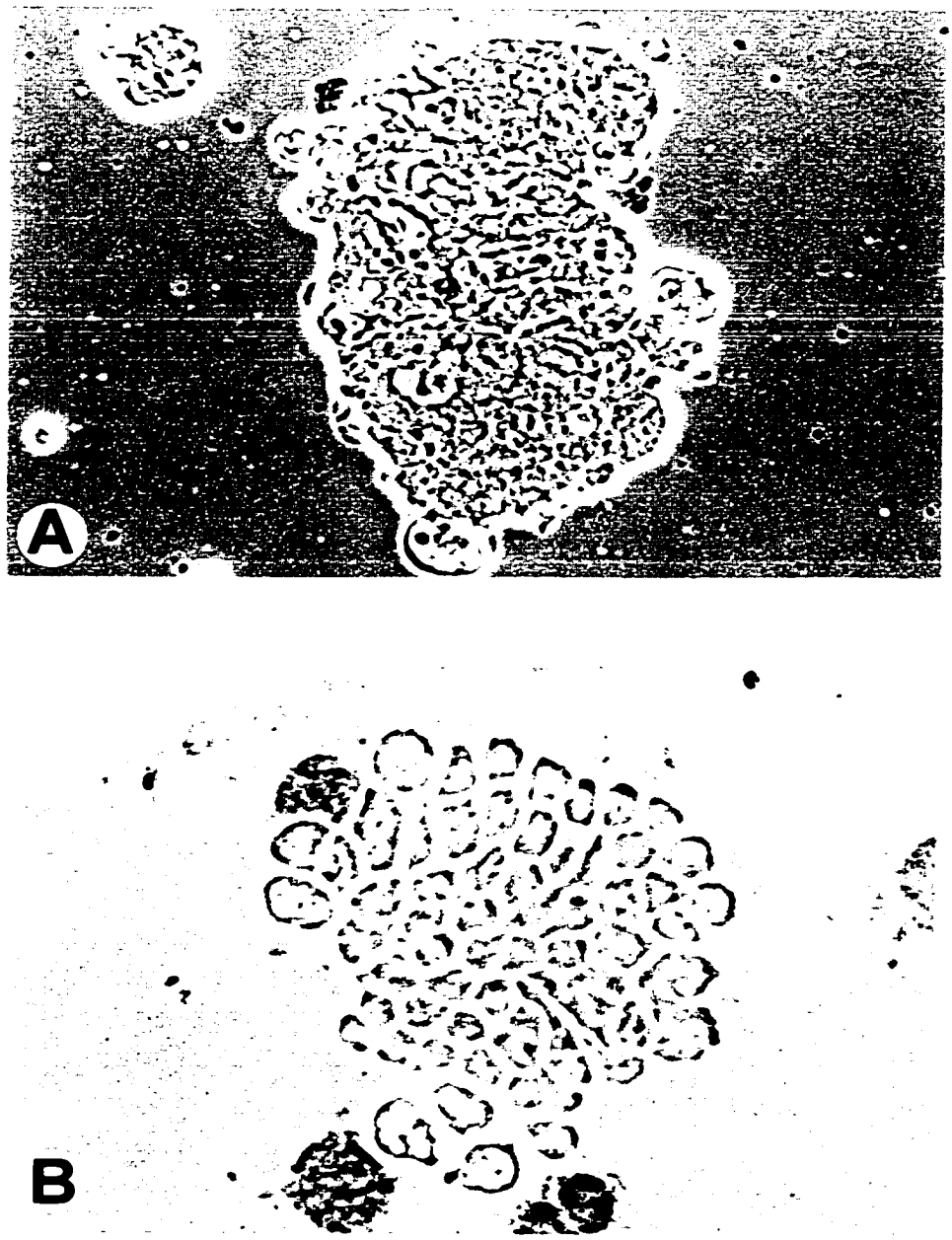


FIG. 6.2: Assessment of membrane integrity of the RBCV-induced polykaryons by trypan blue exclusion.

Polykaryons with intact outer membranes (A) were observed at 12 h postinfection with RBCV-OK-0514-3, cellular aggregates (B) of uninfected G clone cells (× 106).

trypan blue penetrated into cellular compartments. Polykaryons treated with methanol permitted universal uptake of trypan blue (data not shown). Cellular aggregates of uninfected G clone cells maintained distinct contours of individually separated cells (Fig. 6.2B). The large rafts of polykaryons, which were observed with phase-contrast microscopy, consisted of aggregates of smaller polykaryons (Fig. 6.3A). Surprisingly, most of the nuclei in the polykaryons were degenerated, and the remaining intact nuclei were dislocated into peripheral cytoplasmic areas. The uninfected G clone cellular aggregates were stained as distinct cellular entities with defined plasmalemma, cytoplasmic matrices and individual nuclei (Fig. 6.3B).

Membranes of polykaryons fixed with paraformaldehyde had intense immunofluorescence of coronavirus S glycoprotein antigen detected by anti-S Mab 43C2 (data not shown). Cytoplasmic fluorescence of similar intensity was evident after staining acetone-treated polykaryon preparations with this antibody. Fifty percent of the cells, which had not been recruited into polykaryons at 12 h postinoculation, had similar membrane and cytoplasmic fluorescence as detected with anti-S Mab. These cell preparations contained predominantly single cells, but few small fluorescing polykaryons with 2 to 4 nuclei were also observed. Tests with the polycloncal antiserum 1745 gave identical results.

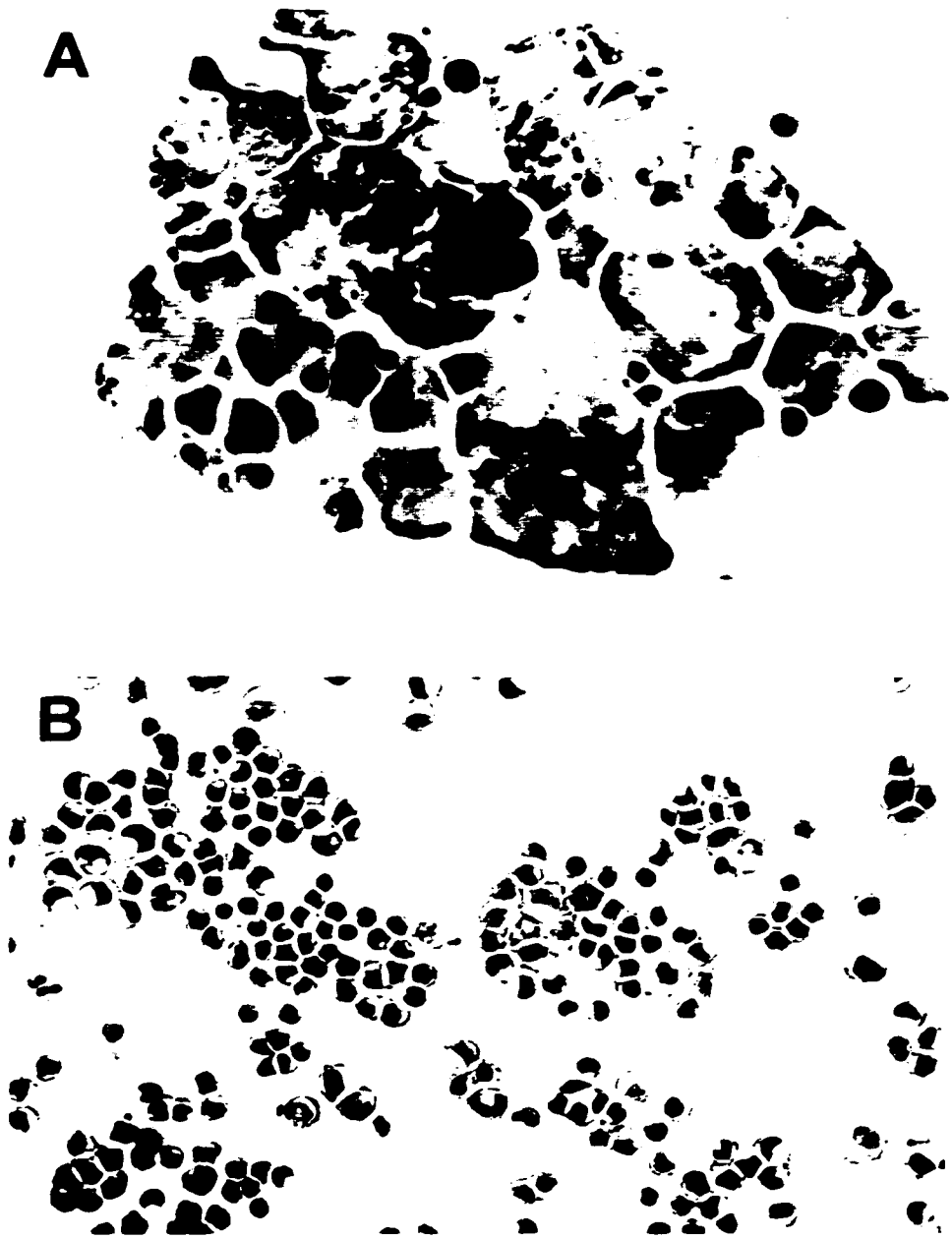


FIG. 6.3: Structural features and morphology of nuclei in RBCV-induced polykaryons after Giemsa staining.

Multiple nuclei were present in peripheral areas of polykaryons (A) induced by RBCV-OK-0514-3 at 12 h postinfection, cellular aggregates (B) of uninfected G clone cells (× 84).

6.3.3 Ultrastructural Features of RBCV-induced Polykaryons

Polykaryons contained multiple, lobulated and misshaped nuclei with fuzzy margins, intense chromatin condensation and margination in conjunction with nuclear membrane separation (Fig. 6.4A). Round RBCV particles, measuring 70 to 80 nm in diameter, were observed in the cytoplasmic matrix of polykaryons (Fig. 6.4B). Two morphological viral structures were found: one consisted of electron-dense cores surrounded by narrow electron-lucent, fringed membranes, and the other was represented by cores with electron-lucent centers creating doughnut-like shapes. Occasionally, smooth-surfaced cytoplasmic vesicles and rough endoplasmic reticula were internally linked with fringed viral structures. Virion morphogenesis began with focal electron-dense thickening on the membranes and by budding into the cisternae of smooth-membraned vesicles. Well-defined, round structures of differing electron densities and varying sizes were in the cytoplasm of polykaryons, some of them contained dense core structures embedded in the matrices, while others did not. Granulofibrillar areas of lower electron-density were also observed. Increasing numbers of swollen mitochondria that had lost their cristae aggregated in perinuclear areas of the cytoplasm.

6.4 Discussion

The highly polarized epithelioid G clone of the parent HRT-18 cells (Lin et al., 1997b) facilitated successful isolation of RBCV strains from nasal swab and pneumonic

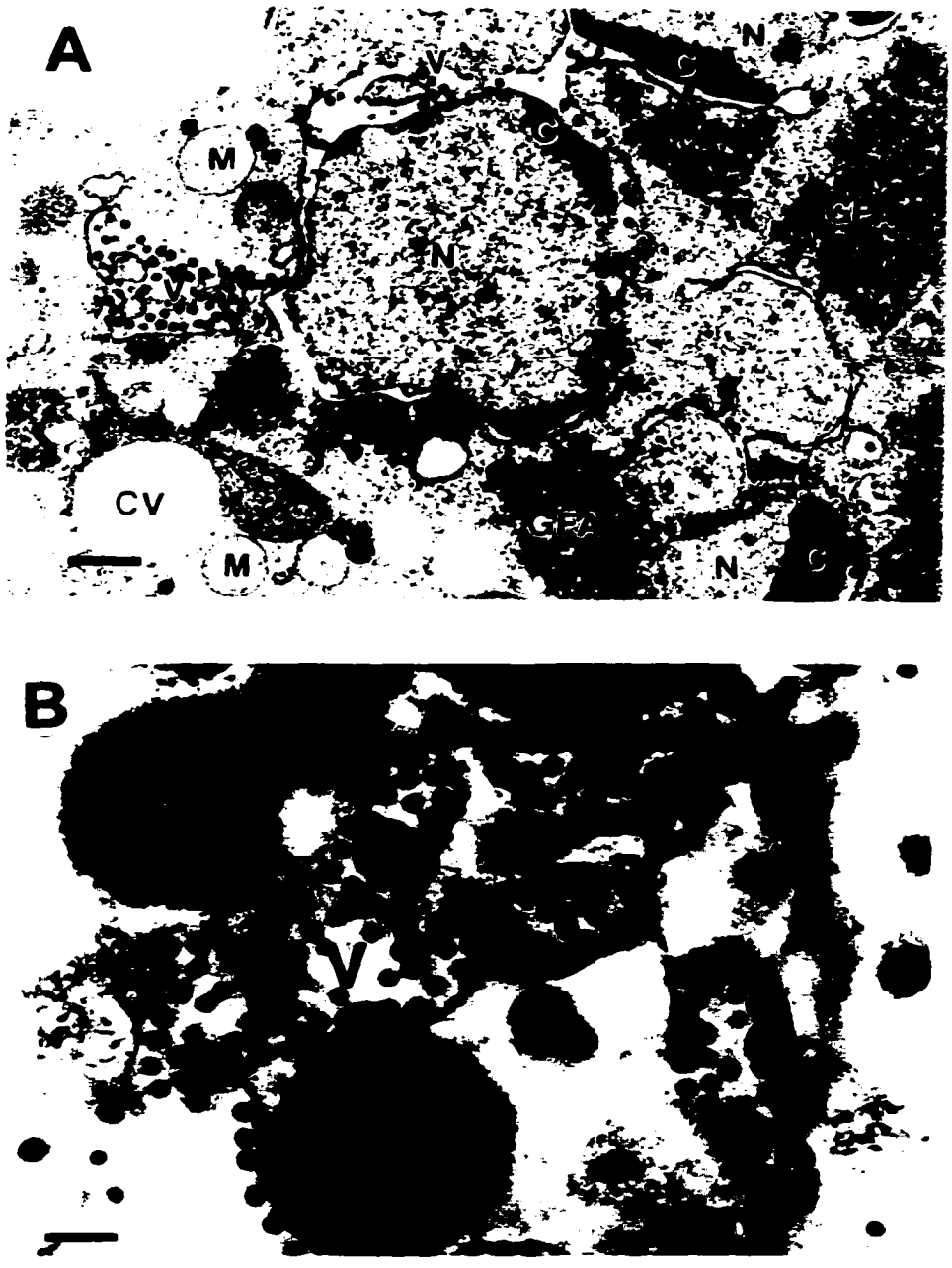


FIG. 6.4: Ultrastructural evidence for RBCV morphogenesis within RBCV-induced polykaryons at 12 h postinfection.

Chromatin margination (C), cytoplasmic vacuoles (CV), granulofibrillar areas (GFA), mitochondria (M), nucleus (N) and virions (V). Uranyl acetate-lead citrate stain (A: × 18,750, B: × 56,250); bar = 0.5 μm (A), = 0.17 μm (B).

lung samples of cattle with acute respiratory tract diseases. The newly isolated RBCV replicated and induced extensive cell fusion in the 1st passage in G clone cells without trypsin enhancement. The trypsin-independent fusogenic activity of RBCV emerges as an important characteristic that distinguishes them from EBCV (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). The EBCV cause enteritis by infecting enterocytes in the intestinal tracts of calves where trypsin or other proteolytic enzymes are present in functional quantities. The RBCV target not only cells of bronchi but also cells of broncheoli and parenchyma of lungs of cattle. Proteolytic enzymes in respiratory tracts do not have physiological roles in digestion processes as they function in the intestinal tracts (Cunningham, 1997). A trypsin-like serine protease was found along surfaces of the trachea and lower respiratory tract of some mammalian hosts, and is theorized to facilitate proteolytic cleavage of hemagglutinin for the influenza virus entry into epithelial cells along respiratory tracts, however, orthomyxovirus infections had not been detected in cattle and other ruminants species (Rott, 1979). Increasing evidence implicates that RBCV infections play etiological roles in acute respiratory tract disease of cattle (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). These RBCV infections pathogenetically are comparable to acute respiratory tract disease induced by influenza virus infections in human subjects, horses, swine, and poultry (Murphy and Webster, 1996; Rott, 1979).

The RBCV-induced polykaryons lost anchorage to substrates and detached from the G clone cell monolayers in synchrony with fusion. The close intercellular associations between the epithelioid HRT-18 cells were destroyed during the polykaryon formation (Payne and Storz, 1990). This phenomenon differs from the behavior of polykaryons induced by murine or porcine coronaviruses in other cell systems which remain attached to substrates (Lai, 1990; Spaan et al., 1988; Wege et al., 1982).

Immunofluorescence techniques were used to correlate expression of viral antigens with cell fusion observed through phase-contrast microscopy. Cytoplasmic membrane expression of S antigens was predominant in the floating RBCV-induced polykaryons. Previous studies of EBCV-L9 infections indicated that EBCV antigens were expressed at the surfaces of infected HRT-18 and fibroblastic BFS cells and that viral antigens appeared in plasma membranes several hours prior to the onset of trypsin-enhanced cell fusion (Payne et al., 1990a and b). Our observations implied that RBCV replication and S antigen expression facilitate formation of polykaryons and adversely affect intercellular adhesion molecules, leading to their loss of anchorage to the substrates.

The viral structural protein responsible for trypsin-activated polykaryocytosis was expressed on the plasma membrane of HRT-18 cells infected with EBCV-L9 strain, and was identified as S glycoprotein (Payne et al., 1990a; St. Cyr-Coats et al., 1988b). The internal sequence KRRSRR was recognized as the probable protease-active site which cleaves S precursor into N-terminal S1 and C-terminal S2 subunits (Abraham et al.,

1990b). The internal hydrophobic domains and heptad repeat region of S2 were found to facilitate cell fusion by forming the coiled-coil structure of the oligomeric S protein (Yoo et al., 1991).

Comparisons of S genes specified by RBCV (OK-0514, LSU-94LSS-051) and EBCV (LY138, Mebus) strains (Chouljenko et al., 1998b) indicated that most of the RBCV-specific nucleotide differences were concentrated on the S1 subunit and resulted in an amino acid substitution within the signal sequence and 2 clusters of amino acid changes within the N-terminus and the hypervariable region. Although the proteolytic cleavage site of the S precursor was conserved among RBCV and EBCV strains analyzed, amino acid changes of Ala 769 to Ser, and Asp 1026 to Gly within the S2 subunit were found to be located next to the proteolytic cleavage site and within the heptad repeat, respectively. We hypothesize that these mutations alone or in conjunction with other RBCV-specific amino acid changes may create conformational changes important for exposing the fusogenic domains of the S2 subunit and for facilitating the high cell-fusing activity of these RBCV isolates.

Paramyxoviruses induced cell fusion at physiological pH ranges among cells infected at high multiplicity (fusion from without) or among cells infected at low multiplicity leading to viral replication (fusion from within) (Hsu et al., 1982). The fusion (F1) protein of the paramyxovirus envelope triggers this membrane fusion activity. Ultrastructural evaluation of RBCV-induced polykaryons identified virions

containing electron-dense cores as well as doughnut-like virus forms in the cytoplasmic matrix, within well-defined cytoplasmic structures of high electron density, membrane-bound cytoplasmic vesicles and smooth endoplasmic reticula. Virions were not seen on the outer surfaces of cytoplasmic membrane of detached polykaryons. Therefore, it is concluded that RBCV-induced polykaryons result from viral replication and thus represent fusion from within.

Floating, RBCV-induced polykaryons had intact plasma membranes with marginated multiple nuclei as indicated by trypan blue exclusion. The plasmalemma maintained integrity for several hours after detachment. Progressive lysis of polykaryons followed gradual membrane and mitochondrial damage as well as karyorrhexis. Cellular aggregates of control G clone cells consisted of individual cells with distinct plasma membranes and nuclei. The characteristic morphological changes observed in RBCV-induced polykaryons included loss of membrane integrity and nuclear degeneration with extensive chromatin condensation and margination. These findings may imply that viral replication within the RBCV-induced polykaryons initiate and accelerate apoptotic processes as mechanisms by which cell death occurs. Further assessment of DNA fragmentation, morphological changes, and changes in cell surface markers should relate the changes in polykaryons to events in apoptosis.

CHAPTER 7

HEMAGGLUTININ-ESTERASE SPECIFIED BY RESPIRATORY BOVINE CORONAVIRUSES HAS TEMPERATURE-SENSITIVE ACETYLESTERASE ACTIVITY

7.1 Introduction

Bovine coronavirus (BCV) is a member of the *Coronaviridae* family, and possesses a single, positive-stranded RNA genome of about 31 kilobases in length (Spaan et al., 1988; Wege et al., 1982). Similar to coronaviruses in antigenic group II, this virus contains a 5th structural protein, the hemagglutinin-esterase (HE), in addition to the nucleocapsid (N), membrane (M), envelope (E) and spike (S) proteins. This 140-kilodalton (kDa) glycoprotein is a disulfide-linked dimer of 2 identical 65-kDa glycosylated subunits, and thus belongs to 65-kDa class I membrane protein (Brian et al., 1995). It forms the short peplomere on the viral envelope (Brown and Brierly, 1995; Deregt et al., 1987; Hogue et al., 1989).

The BCV HE serves as a receptor-binding and receptor-destroying glycoprotein similar to the hemagglutinin-esterase-fusion (HEF) glycoprotein of influenza C virus (Deregt et al., 1987; Herrler et al., 1988; Hogue et al., 1984; King et al., 1985; Strobl and Vlasak, 1993; Vlasak et al., 1988a and b, 1989). It binds to the N-acetyl-9-O-acetylneuraminic acid residues of glycoproteins or glycolipids on the surfaces of erythrocytes and susceptible cells, which is considered to be the major receptor

determinant of BCV (Herrler et al., 1985; Herrler et al., 1991; Vlasak et al., 1988a). The receptor-destroying enzyme (RDE) activity of BCV HE is the first example of such an enzyme identified on positive-stranded RNA viruses (Vlasak et al., 1988a and b). Enzymes with receptor-destroying functions had been identified as major structural components of negative-stranded RNA viruses such as orthomyxoviruses and paramyxoviruses (Hirst, 1950). The receptor-destroying functions are mediated by neuraminidases (NA) of influenza A and B viruses as well as paramyxoviruses which remove terminal N-acetylneuraminate from glycoconjugates (Klenk et al., 1955). In contrast, RDE of both influenza C virus and BCV have acetylesterase (AE) activities which hydrolyze an ester linkage to release the acetyl group from position C-9 of N-acetyl-9-O-acetylneuraminic acid, potentially eluting adsorbed virions (Herrler et al., 1985; Vlasak et al., 1988a and b). The 9-O-acetyl residue is important for influenza C virus recognition of a glycoprotein receptor, a major determinant for the cell tropism (Herrler and Klenk, 1987).

Inhibition of the AE activity of BCV HE by diisopropylfluorophosphate resulted in some reduction of viral activity, indicating that binding of HE to N-acetyl-9-O-acetylneuraminic acid residue involved in infectious process (Vlasak et al., 1988a). Enzymatic removal of the N-acetyl-9-O-acetylneuraminic acid residue from cell membranes or treatment with HE-specific monoclonal antibodies inhibits BCV infections (Deregt et al., 1989; Schultze and Herrler, 1992). Additionally, cells infected

with a recombinant baculovirus expressing the HE of BCV exhibited hemadsorption and esterase activities, both of which could be blocked by monoclonal antibodies with infectivity neutralization activity (Parker et al., 1990; Yoo et al., 1992). Consequently, it appears that the HE of BCV is important in virus infectivity. In addition to the S glycoprotein which has strong receptor-binding properties (Schultze et al., 1991a and b; Schultze and Herrler, 1992), binding of HE glycoprotein of the short peplomeres to N-acetyl-9-O-acetylneuraminic acid residue on the cell surface may function as a prereceptor interaction for BCV. However, the HE of mouse hepatitis virus strains A59 and JHM is not expressed in productive infections, and thus is not essential for replications of these strains (Shieh et al., 1989).

Numerous coronaviruses recently were isolated from nasal swab samples and lung tissues of feedlot and other cattle with signs of acute respiratory tract disease including a severe form of shipping fever pneumonia (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). These virus isolates multiplied only in the G clone of human rectal tumor-18 (HRT-18) cells, and not in cultured bovine cells, such as Georgia bovine kidney (GBK) and bovine turbinate (BT) cells which were permissive for the known respiratory bovine viruses including bovine herpes virus-1 (BHV-1), bovine parainfluenza type-3 virus (PI-3), bovine respiratory syncytial virus (BRSV) or bovine viral diarrhea virus (BVDV). These viruses were identified as respiratory bovine coronaviruses (RBCV) based on their morphological features as determined by electron

microscopy, their ability to induce cell-to-cell fusion after infection of G clone cells, and the nucleotide (n.t.) sequence of structural genes. Phenotypic and genotypic properties of RBCV differentiated them from the previously reported enteropathogenic bovine coronaviruses (EBCV) (Chouljenko et al., 1998b; Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). Distinguishing features are: (1) The RBCV were isolated in the 1st G clone cell passage at neutral pH ranges without the use of trypsin enhancement. Trypsin activation was required for the isolation of EBCV (Storz et al., 1981). (2) The RBCV have unusually high cell-fusing activities for the G clone cells. (3) The RBCV have a restricted hemagglutination pattern, and agglutinate only mouse and rat, but not bovine and chicken red blood cells (RBC). The EBCV agglutinates both rodent and chicken RBC (Storz et al., 1992). (4) Comparative analysis of wild-type RBCV and EBCV n.t. and amino acid (a.a.) sequences at the 3' genomic portion (9.5 kilobases) revealed that RBCV-specific n.t. and a.a. changes were disproportionally concentrated within the HE gene, S gene and the genomic region between the S and E genes (Chouljenko et al., 1998b). Most of these RBCV isolates have RDE activities for rat RBC (Lin et al., 1995).

We hypothesized that differences in structural and functional properties of HE and other BCV structural proteins may cause altered tissue tropism and virus pathogenicity. The objectives of this investigation were to identify the enzymatic reaction associated with the RDE function of HE for RBCV, to characterize the AE activities of HE

glycoproteins from wild-type strains of RBCV and EBCV in purified virus preparations and as transiently expressed gene products at specific body temperatures, and to compare their HE cDNA-predicted a.a. sequences.

7.2 Materials and Methods

7.2.1 Virus Strains and Virus Purification

The wild-type strains of RBCV, OK-0514 (OK) and LSU-94LSS-051 (LSU), were used at their 2nd to 4th passages. The EBCV wild-type strain LY138 (LY) was used at its 2nd and 3rd passages. All viruses were propagated in the G clone cell. Virus purification was performed according to Zhang et al. (Zhang et al., 1994). Briefly, the media of infected G clone cell cultures were clarified by low speed centrifugation following 3 freezing and thawing cycles. Supernatant fluids were collected and precipitated by 10% (w/v) polyethylene glycol. Suspensions of precipitates were loaded onto a 20% sucrose cushion, and further purified by a 20 to 60% sucrose gradient. The purified virus preparations were resuspended in TNE buffer (100 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4) and stored at −70°C. Virus infective titers in the preparations for RBCV OK and LSU, and EBCV LY ranged from 3.0 to 4.0 $\times$ 10^9 plaque forming units (PFU)/ml.

7.2.2 Assays for Hemagglutinin (HA) and RDE

The assays for HA and RDE were performed as reported (Storz et al., 1999; Storz et al., 2000a and b) with washed rat RBC prepared as suspensions of 0.5% with PBS (pH7.4), containing 0.05% bovine serum albumin.

7.2.3 Cloning and Sequencing of the cDNA Specifying the HE Glycoprotein and Alignment of Predicted Amino Acid Sequences of Different HE Genes

Viral RNA extraction, cDNA synthesis and DNA sequencing were performed as reported (Chouljenko et al., 1998b), using TRI reagent (Molecular Research Center, Inc., Cincinnati, Oh.), Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech Inc., Uppsala, Sweden), PCR reagents, AmpliTaq and Gene-Amp PCR system 9600 (Perkin-Elmer, Norwalk, Conn.), TA-cloning kit (Invitrogen Inc., San Diego, Calif.), restriction enzymes (New England Biolabs, Beverly, Mass.), and sequenase™ (Amersham Life Science, Arlington Heights, Il.). A cDNA HE fragment was amplified by polymerase chain reactions (PCR) using a specific primer pair 5F16/3B10 which corresponded to the sequences upstream and downstream of HE gene, respectively. The actual primer sequences are: 5F16: 5'AGAATGGCAGTTGCTTATG3'; 3B10: 5'GATTATGGTCTAAGCATCATG3'. The 1,272-base pair (bp) DNA fragment obtained by RT-PCR was first cloned into the pCR™ II vector and subsequently cloned into the unique Not I- and Hind III- restriction endonuclease sites of the eukaryotic expression plasmid vector pcDNA3.1/Zeo(+) (Invitrogen Inc.). The predicted a.a.

sequences of the HE genes from EBCV LY, RBCV OK and LSU were aligned with the aid of the Sequence Analysis Software Package version 6.1 of the Genetics Computer Group of the University of Wisconsin and the MacVector Software (IBI, New Haven, Conn.).

7.2.4 DNA Transfection and Transient Expression of BCV HE Genes in COS-7 Cells

The HE genes were transfected into COS-7 cells using Lipofectamine™ (Life Technologies Inc., Gaithersburg, Md.). Eighteen to 24 h prior to transfection, COS-7 cells were plated at an appropriate cell density to obtain approximately 50 to 80% confluency the next day. Two μg of plasmid DNA and 10 μl of lipofectamine reagent were mixed for each transfection, and incubated for 30 min at room temperature to form DNA-liposome complexes which were placed onto the cells grown in a 35-mm well together with serum and antibiotics-free NTCT medium (Sigma Inc., St. Louis, Mo.). After incubation at 37°C for 7 to 9 h, the transfection mixture was replaced with DMEM (Sigma Inc.) with 10% fetal bovine serum, and the cells were further incubated for another 60 h. When transient expression of HE was assessed by indirect immunofluorescence assay (IFA), 0.5 μg of plasmid DNA and 2.5 μl of Lipofectamine™ were applied to COS-7 cells grown on a 12-mm coverslip (Fisher Scientific, Pittsburgh, Pa.). Then the transfected cells were fixed with methanol at 60 h post transfection (h.p.t.), incubated with polyclonal antiserum from BCV-infected cattle

(Storz and Rott, 1980; Storz and Rott, 1981), and reacted with fluorescien isothiocyanate (FITC)-labeled affinity purified goat anti-bovine IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.), counter-stained with Evans blue, and examined with a fluorescence microscopy (Olympus AH-2, Olympus Optical Co. LTD, Japan).

7.2.5 Acetylesterase Assay for Acetate Release from Bovine Submaxillary Mucin (BSM)

Cells in six 35-mm wells were transfected with each plasmid DNA as described above, and used for the AE activity according to Herrler at al (Herrler et al., 1985). Briefly, the cells were scrapped off after 69 h.p.t., washed with phosphate-buffered saline (PBS) and resuspended in 0.6 ml PBS. The cellular suspension was treated with sonication at a power setting of 5.5 for 10 pulses (Branson Sonifier Cell Disruptor 200, Branson Ultrasonics Co., Danbury, Conn.), and soluble proteins were collected from supernatant after low speed centrifugation. The supernatant amount was mixed in 30 µl with 50 µl BSM (type I-S, Sigma; 25 mg/ml in PBS), and incubated at 37°C and 39°C, while 10 µl of purified virus preparation was used. The mixture was assayed at specific time points for the presence of acetate with a commercial test kit (Boehringer-Mannheim Inc., Indianapolis, Ind.). Reaction controls consisted of a sample of BSM and the same amount of the supernatant from transfected COS-7 cell lysate or purified

virion preparation which reacted at 4°C. The values of the control samples were subtracted from those of the test samples.

7.2.6 Serine-esterase Assay for Nitrophenol Release from ρ-nitrophenyl Acetate (PNPA)

The serine-esterase activity was determined according to Vlasak et al (Vlasak et al., 1988a). Thirty-five µl of the soluble protein preparation or 5 µl of purified virus preparation were incubated with 1 mM PNPA in PBS at room temperature. The release of nitrophenol was measured at 405 nm for 10 min at intervals of 1 min. A sample of the PNPA incubated with untransfected or uninfected cellular preparation was used as the background level, which was subtracted from the test samples.

7.3 Results

7.3.1 Analysis of HA and RDE Activities of RBCV and EBCV

The HA and RDE activities of RBCV and EBCV were analyzed before and after virus purification, as shown in Table 7.1. Virus preparations of RBCV and EBCV agglutinated rat RBC with HA titers ranging from 64 to 128 per 50 µl, and their associated RDE titers were 4 to 8 before purification. The specific HA and RDE titers of the virus preparations increased to titers of 4096 and 1024, respectively, after the virus purification procedure.

Table 7.1: Hemagglutinin and Receptor-destroying Enzyme Activity of Respiratory and Enteropathogenic Bovine Coronaviruses

		HA Titer	RDE Titer
Before purification			
	RBCV-OK-0514-3	64	4
	RBCV-LSU-94LSS-051-2	128	8
	EBCV-LY138-3	128	8
After purification			
	RBCV-OK-0514-3	2048	512
	RBCV-LSU-94LSS-051-2	4096	1024
	EBCV-LY138-3	4096	1024

RBCV: respiratory bovine coronavirus; EBCV: enteropathogenic bovine coronavirus; Hemagglutinin (HA) titers were expressed as hemagglutination units (HAU)/50 μl, reflecting the highest titer with complete agglutination; Receptor-destroying enzyme (RDE) titers were indicated as the highest dilutions with complete elution of adsorbed virions, that was the deaggregation of previous aggregation.

7.3.2 Transient Expression of HE on Transfected COS-7 Cells

The HE genes from RBCV OK and LSU, and EBCV LY were cloned into the transient expression vector pcDNA3.1/Zeo(+) under the control of cytomegalovirus immediate early promoter (pCMV) and transiently expressed in COS-7 cells (Fig. 7.1A). All 3 HE proteins were expressed in similar amounts as indicated by the IFA with BCV-specific polyclonal antibody. Fluorescent signals were detected in the cytoplasm and perinuclear sites of approximately 5% of COS-7 cells at 69 h.p.t. (Fig. 7.1B).

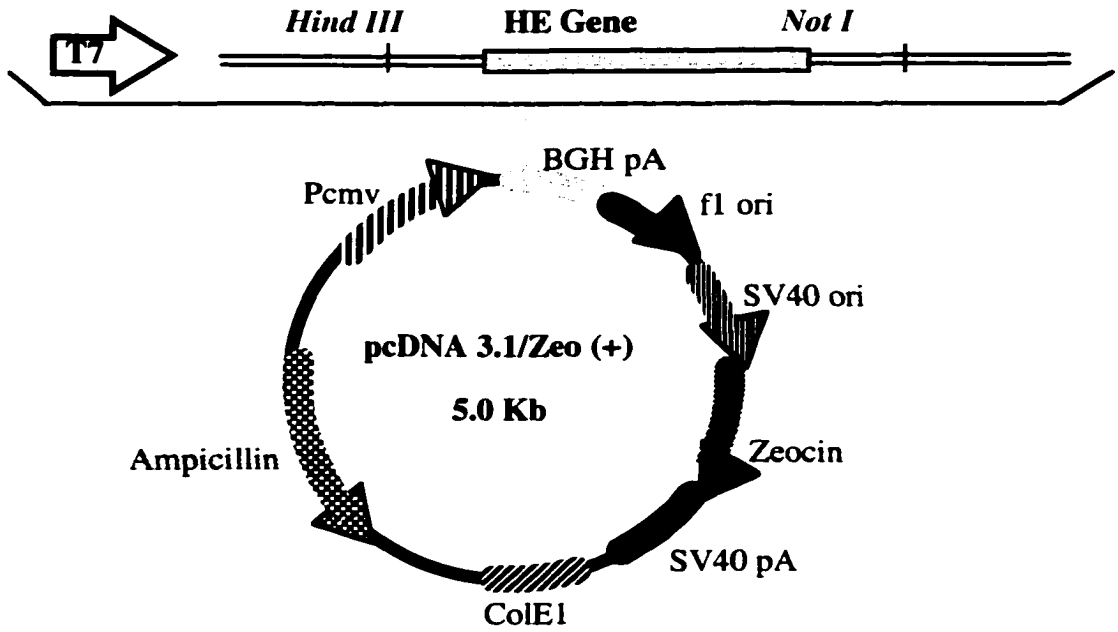
7.3.3 Serine-esterase and AE Enzymatic Activities of Purified Virus Preparations and Extracts of Transfected COS-7 cells

High levels of serine-esterase activity were detected in purified virus preparations at room temperature using the synthetic serine-esterase substrate PNPA (Fig. 7.2A). The RBCV OK and LSU, and EBCV LY virus preparations with identical PFU titers released equivalent amounts of nitrophenol from PNPA. The COS-7 cellular extracts containing HE specified by RBCV OK and LSU, and EBCV LY strains hydrolyzed PNPA effectively (Fig. 7.2B). The COS-7 cellular extracts containing HE of RBCV OK exhibited a lower rate of time-dependent serine-esterase activity than those of RBCV LSU and EBCV LY.

FIG. 7.1: Cloning and expression of HE gene in COS-7 cells.

(A) The PCR-amplified HE gene was cloned into pcDNA3.0/Zeo(+) vector. (B) Expression of HE genes from RBCV-OK-0514-3 (a), RBCV-LSU-94LSS-051-4 (b), and EBCV-LY138-2 (c) was detected after transfection into COS-7 cells using indirect immunofluorescence assay. The G clone cells infected with RBCV-OK-0514-3 were used as positive control (d).

(A)



(B)

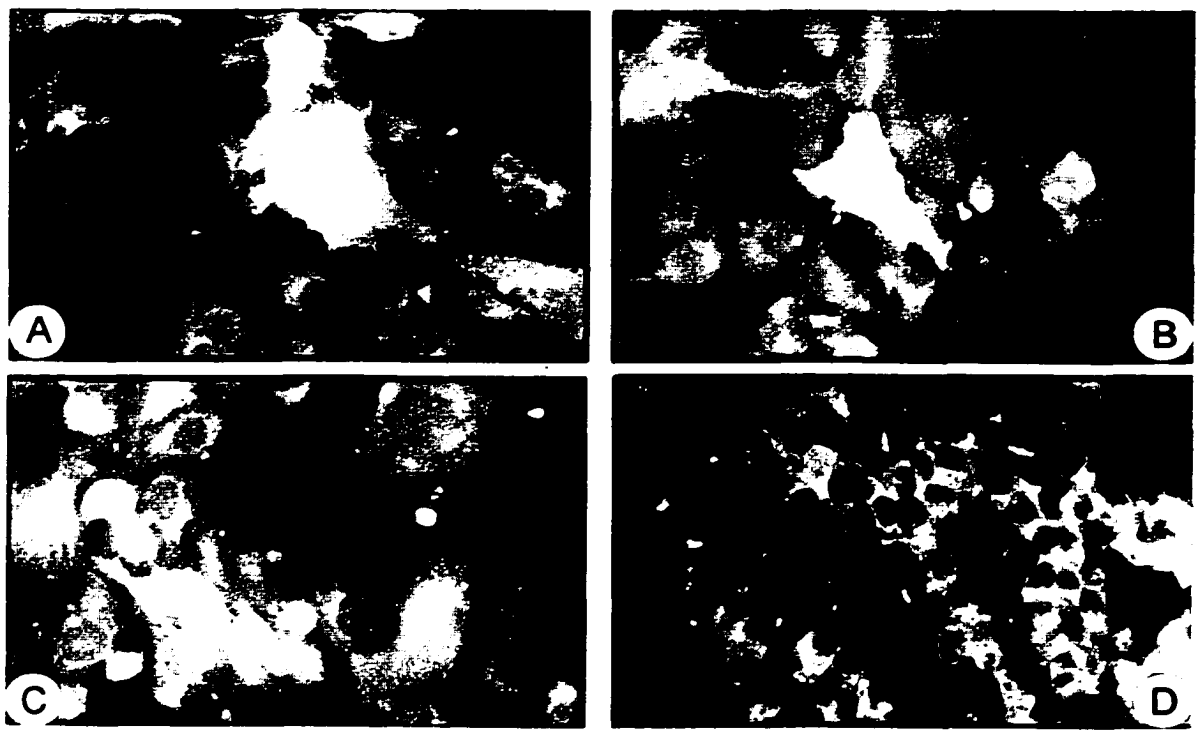
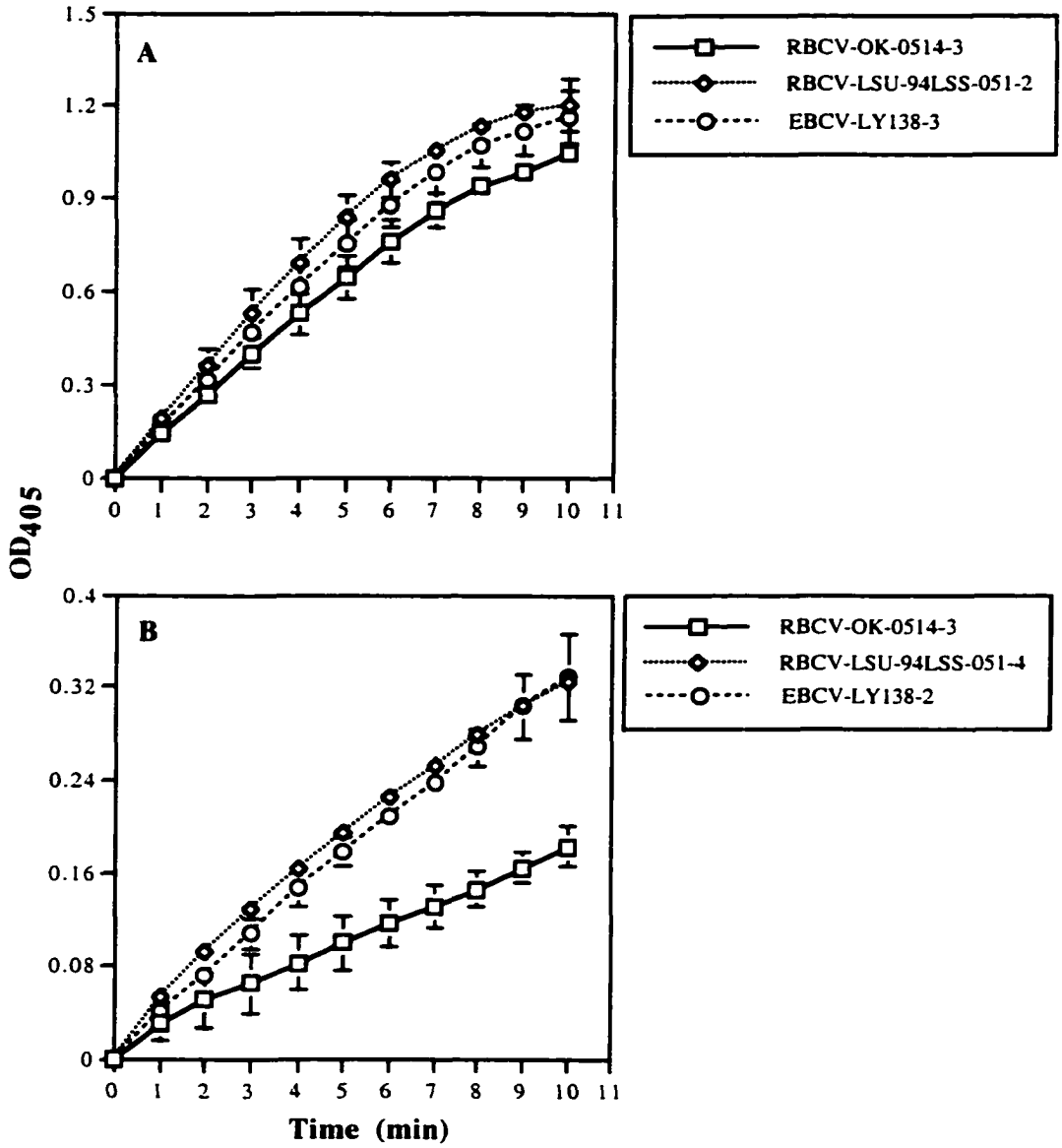


FIG. 7.2: Serine-esterase activities associated with purified RBCV and EBCV, and expressed HE proteins from RBCV and EBCV.

ρ-nitrophenyl acetate was incubated at room temperature with purified RBCV-OK-0514-3, RBCV-LSU-94LSS-051-2, and EBCV-LY138-3 (A); and expressed HE proteins from RBCV-OK-0514-3, RBCV-LSU-94LSS-051-4, and EBCV-LY138-2 (B). Data are means ± standard errors of means (n=3).



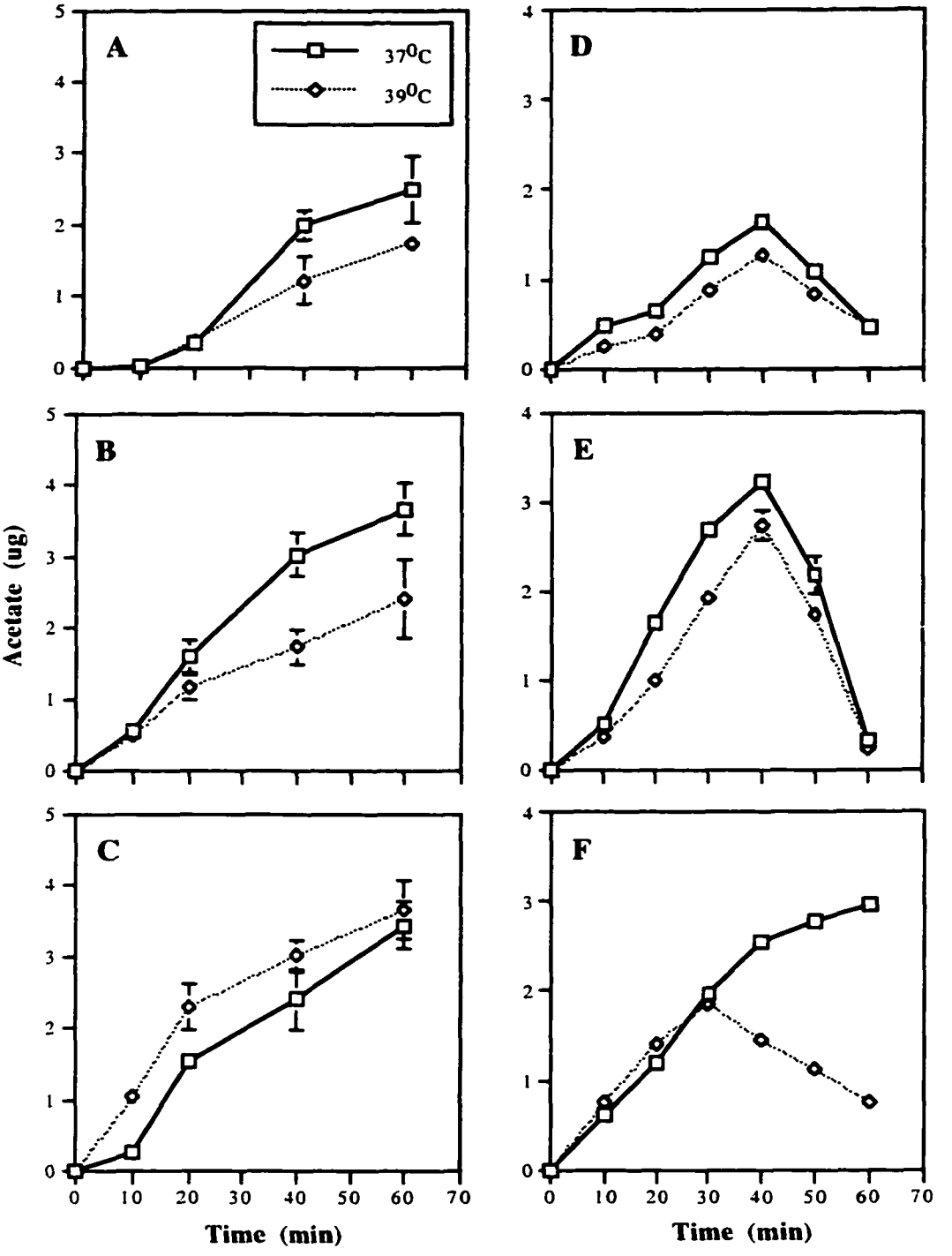
The AE functions of HE proteins were further tested in a 60 min-assay by monitoring the release of acetate from a natural esterase substrate, BSM, at 37°C and 39°C (Fig. 7.3). Purified preparations of RBCV OK and LSU, and EBCV LY released similar amounts of acetate over time at 37°C (Fig. 7.3A, 7.3B and 7.3C). Purified EBCV-LY preparation hydrolyzed 9-O-acetylated sialic acids in BSM at a higher level at 39°C than it did at 37°C. In contrast, purified RBCV OK and LSU preparations had reduced AE activities at 39°C in comparison to the AE activities at 37°C. Cellular extracts from COS-7 transfected with HE genes were also assayed for their AE activities. At 37°C, release of acetate from BSM by these COS-7 cellular extracts containing the HE of EBCV LY increased for up to 60 min (Fig. 7.3F). In contrast, the ability of expressed HE of RBCV OK and LSU to hydrolyze 9-O-acetylated sialic acids in BSM at this reaction temperature increased over time for 40 min, and subsequently declined to minimal levels of activities at 60 min (Fig. 7.3D and 7.3E). The expressed HE of EBCV LY rapidly decreased acetate liberation after 30-min reaction time at 39°C, while the AE activity of the expressed HE of RBCV OK and LSU was further reduced after 40 min (Fig. 7.3D, 7.3E and 7.3F).

7.3.4 Comparison of HE cDNA-predicted A.A. Sequences Specified by RBCV and EBCV

Comparison of the deduced a.a. sequences of RBCV OK and LSU, and EBCV LY strains revealed specific a.a. differences in HE (Fig. 7.4). The HE a.a. sequence of

FIG. 7.3: Acetate release from bovine submaxillary mucin (BSM) by purified RBCV and EBCV, and expressed HE proteins from RBCV and EBCV.

Purified RBCV-OK-0514-3 (A), RBCV-LSU-94LSS-051-2 (B), EBCV-LY138-3 (C); and expressed HE proteins from RBCV-OK-0514-3 (D), RBCV-LSU-94LSS-051-4 (E), and EBCV-LY138-2 (F) were incubated with BSM at 37°C (□) and 39°C (◊). Data are means ± standard errors of means (n=3).



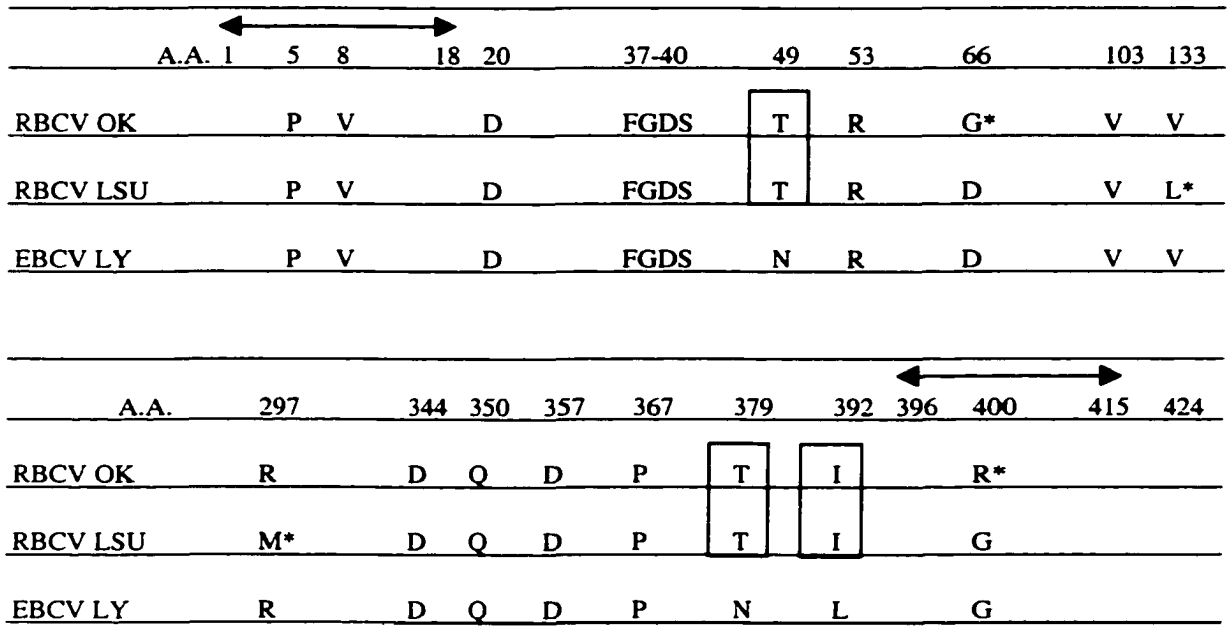


FIG.7.4: Comparison of the predicted amino acid sequences of the HE proteins specified by different bovine coronavirus strains.

The compared strains included newly isolated respiratory bovine coronavirus (RBCV) strains OK and LSU, and prototype enteropathogenic BCV (EBCV) wild-type strain LY, Amino acids (a.a.) 1-18 are the predicted signal peptide, and a.a. 396-415 are the predicted intramembrane-anchoring sequence. *demarcates RBCV strain-specific a.a. changes in comparison to the EBCV LY. Boxed a.a. are unique for RBCV.

EBCV LY differed from those of both RBCV OK and RBCV LSU at positions 49, 379 and 392. Both a.a. substitutions at positions 49 and 379 were from Asn (LY) to Thr (OK, LSU), while the a.a. substitution at position 392 was from Leu (LY) to Ile (OK, LSU).

7.4 Discussion

We examined and compared the structure and function of HE glycoproteins specified by RBCV and EBCV strains. We documented that the HE of RBCV had AE activity, which mediated the RDE function, a characteristic of BCV. Moreover, we showed that the enzymatic activities of HE could be measured by transient expression of HE genes in COS-7 cells. This transient expression system allows the functional characterization of HE proteins in the absence of any other BCV components. We found that the HE of RBCV can be differentiated from that of EBCV on the basis of temperature sensitivity, and identified unique RBCV-specific a.a. changes, which may cause the observed AE enzymatic activities.

Overall, the serine-esterase activity assayed by the PNPA method indicated that transiently expressed HE was enzymatically active, but this assay did not detect any differences between HE of RBCV and EBCV. In contrast, the AE activity tested with BSM as substrate, which mimics the natural enzymatic function of HE, indicated that the AE activity of RBCV-specified HE obtained from either purified virus preparations or through transient expression in COS-7 cells was more temperature-labile than that of

EBCV. Specifically, the AE activities of purified EBCV LY virus preparations were stable at both 37°C and 39°C, while reduced AE activity of purified RBCV OK and LSU was observed at 39°C as compared with that at 37°C. In contrast, transient expression of HE in COS-7 cells indicated that HE of EBCV LY was stable at 37°C, but was temperature-labile at 39°C, while HE of RBCV OK and LSU was not stable at both 37°C and 39°C. This property infers that HE interaction with other viral structural components may stabilize its structure and enzymatic functions. Multiprotein interaction between S and HE glycoproteins was first suggested by experiments with monoclonal antibodies against HE of EBCV-L9 which inhibited both hemagglutination and AE activities (Storz et al., 1991; Zhang et al., 1991).

The HE cDNA-predicted a.a. sequences specified by RBCV contained 3 a.a. substitutions. Two substitutions constituted nonconservative a.a. changes of the smaller polar Asn to the small nonpolar Thr. These substitutions were located 9 a.a. immediately downstream from the conserved esterase active site ($FGDS_{40}$), and 18 a.a. upstream from the predicted intramembrane-anchoring sequence. The third RBCV-specific a.a. change involved the conservative a.a. substitution of Ile_{392} (RBCV) from Leu_{392} (EBCV). These specific a.a. changes may be responsible for the altered AE activities of RBCV. Future experiments with site-directed mutagenesis should

determine whether these a.a. changes are specifically associated with the function of RBCV HE.

The coronavirus HE genes have 30% a.a. sequence homology with HEF glycoprotein of influenza C virus, and may have been derived by a recombination between an HEF mRNA of influenza C virus and the genomic RNA of an ancestral coronavirus during a mixed infection (Luytjes et al., 1988). Importantly, coronavirus HE has both receptor-binding (Deregt et al., 1987; Hogue et al., 1984; King et al., 1985) and AE-mediated receptor-destroying functions which are similar to the HEF of influenza C virus (Herrler et al., 1985; Herrler et al., 1991; Vlasak et al., 1988a and b). The influenza C virus HEF glycoprotein mediates viral attachment to cells through its receptor-binding activity for 9-O-acetyl-N-acetylneuraminic acid and viral penetration through its fusion activity (Herrler et al., 1988; Strobl and Vlasak, 1993; Vlasak et al., 1989). The AE of this viral protein, which hydrolyzes an ester linkage to release acetate from the viral receptor determinant, 9-O-acetyl-N-acetylneuraminic acid, allowing release of mature virus from infected cells or from erythrocytes.

The receptor-destroying activity of influenza A and B viruses is mediated by an NA which catalyzes the cleavage of the α-ketosidic linkage between a terminal N-acetylneuraminate and an adjacent D-galactose or D-galactosamine, and releases N-acetylneuraminate from the glycoconjugates on the cell surface (Gottschalk, 1957; Palese et al., 1974). Another potential role of NA was suggested to permit transport of

the virus through mucin layer present in the respiratory tract, facilitating the virus interaction with the target epithelial cells. Some NA (N1 and N9 subtypes) have both a receptor-binding site and an NA active site (Hausman et al., 1993; Laver et al., 1984). However, the receptor specificity and function of this receptor-binding activity is still unknown. The precise role of HE glycoprotein in coronavirus evolution, replication and pathogenesis remains a mystery. The coronavirus HE has been suggested to serve as a second viral attachment protein in addition to the S glycoprotein peplomere (Parker et al., 1989; Vlasak et al., 1988b). Theoretically, a remarkable selective advantage is provided by the HE to coronaviruses in infections of mucous membrane. The HE could facilitate virus release and spread, virus migration through the mucus layer, and ultimately virus infection of epithelial cells through its specific and reversible binding to the substrate, cleavage and rebinding.

The EBCV primarily causes severe diarrhea in neonatal calves and winter dysentery of adult cattle by infecting absorptive epithelial cells of the small intestine (Doughri et al., 1976; Mebus et al., 1973; Saif et al., 1988). The temperature at this site equals the body temperature of around 39°C of cattle, typically higher than the temperature of the epithelial surface of air passages which is about 37°C. We hypothesize that observed AE activities of HE described in this paper may directly or indirectly contribute to RBCV infections of respiratory tracts of cattle. The AE of the RBCV strains may have lost their stability at 39°C because these viruses evolved

through replication in respiratory tissues. Conversely, it is possible that conformational changes leading to less thermostable AE activity of the RBCV strains may increase the ability of these viruses to replicate in respiratory tissues. Additional studies are required to resolve these possibilities.

CHAPTER 8

CONCLUSION AND PERSPECTIVES

Respiratory tract diseases remain an economically important problem in the beef and dairy cattle industries of this country. Shipping fever pneumonia represents the most serious component of the bovine respiratory disease complex. This disease is hypothesized to be attributed to multifactorial causes whereby stressful conditions favor initial virus infections of respiratory tracts which are often complicated by bacterial infections, leading to potentially fatal pneumonia (Hoerlein, 1980; Yates, 1982). Numerous attempts had been made to identify etiological infectious agents and their mechanisms of pathogenesis, and to induce typical shipping fever pneumonia by respiratory exposure of cattle for decades (Baldwin et al., 1967; Briggs et al., 1998; Collier, 1968; Friend et al., 1977; Hoerlein et al., 1961; Whitely et al., 1992; Yates, 1982). Although vaccines and antibiotics were developed to protect cattle from respiratory tract infections with known viruses and bacteria, not all bovine respiratory tract disease problems were controlled, and shipping fever pneumonia remains particularly prevalent among beef cattle recently transported to feedlots (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b).

During the last 5 years, a total of 411 coronavirus strains were isolated from respiratory tract samples of 388 cattle in virtual absence of other respiratory virus

infections during the early stages of disease epizootics that were investigated. These cattle originated from 11 different states, and included cattle involved in 3 major shipping fever epizootics of 1993, 1997 and 1998, cattle from livestock shows, hospital cases and cattle on winter pastures. This surprising finding strongly suggests that coronavirus has recently emerged as a major infectious factor of acute respiratory tract diseases in the vaccinated cattle population with apparent control of the known virus infections. The etiological involvement of coronavirus in shipping fever pneumonia and other acute bovine respiratory tract diseases were virtually not recognized in the past because either this virus infection did not exist previously, or proper technologies were not available for detecting this virus infection.

The pathogenic mechanism for the coronavirus infection of respiratory tracts of cattle derived from the unique phenotypic and genotypic properties of this virus. One important phenotypic characteristic of RBCV is its trypsin-independent fusogenic activity in the G clone cells (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). The RBCV-induced polykaryons result from viral replication and S protein expression on plasma membranes. Ultrastructural analysis of the polykaryons may imply the associated apoptotic processes as mechanisms by which cell death occurs. Studies on the genetic properties of RBCV S protein suggest that RBCV-specific amino acid changes may create conformational changes important for exposing the fusogenic domains of the S2 subunit and for facilitating the high cell fusing activity

of RBCV. Another significant property of RBCV is the high AE activity at 37°C which equals the temperature at respiratory tracts of cattle, while the AE function of EBCV is much more active at 39°C, the body temperature of cattle. Restricted apical routes of RBCV entry into or release from highly polarized epithelioid G clone cells are also proposed as potential mechanisms utilized by the virus to circumvent the epithelial barrier *in vivo*. When the tight junctional complexes are intact between the target epithelial cells, RBCV can enter the cells only through apical surfaces, leading to localized infection. However, under pathogenic conditions when junctional complexes detach, RBCV may infect the epithelial cells through both apical and lateral areas, causing potential systemic infection. Recently, evidence implicated that RBCV infections play etiological roles in acute respiratory tract disease of cattle (Storz et al., 1996; Storz, 1998; Storz et al., 1999; Storz et al., 2000a and b). These RBCV infections pathogenetically are comparable to acute respiratory tract disease induced by influenza A virus infections in human subjects, horses, swine, and poultry (Murphy and Webster, 1996; Rott, 1979). Interestingly, orthomyxovirus infections have not been observed in cattle and other ruminants species (Rott, 1979).

Pasteurella haemolytica was the bacterial species predominantly cultivated from pneumonic lung samples of the dead cattle. It was evident that the RBCV infection occurred first. Secondary *P. haemolytica* infection played important and sequentially interactive roles in the pathogenesis of the severe pneumonia of the calves. Respiratory

tract defenses of cattle were weakened by the high loads of RBCV infections through the AE function of HE glycoprotein during the early phase of respiratory tract disease episodes. The HE binds to the 9-O-acetylated neuraminic acid residues of glycoconjugates on the surfaces of target cells and glycocalyx in mucus layer, which is considered to be the major receptor determinant of coronavirus (Herrler et al., 1985; Herrler et al., 1991; Vlasak et al., 1988a and b). The AE activity of HE hydrolyzes an ester bond to release the acetyl group from the substrates, resulting in elution of adsorbed virions and chemical changes in glycokalyx with loss of viscosity. Through this specific and reversible binding, cleavage and rebinding, HE could theoretically not only facilitate virus migration, infection, release and spread but also enhance adhesion of *P. haemolytica* through weakening mucosal resistance barrier.

For the first time, antibody responses of cattle against RBCV infections from the onset of a naturally occurring severe shipping fever epizootic to complete recovery of affected cattle or fatal outcomes were defined. Cattle nasally shedding RBCV at the beginning of the epizootic experienced characteristic primary immune responses with specific antibodies for HE and S proteins. The dead cattle had only IgM responses to RBCV infections. Cattle remaining negative in RBCV isolation tests entered this experiment with antibody against HE and S. Protection against respiratory tract disease was apparently afforded by high levels of opsonic and virus-neutralizing IgG2. The HE and S glycoproteins were recognized earliest by the bovine immune system while the N

protein induced antibody responses during the later stage of initial infection and the early stage of reinfection. The membrane (M) glycoprotein was the least immunogenic of the major viral structural proteins.

Koch's postulates have not been proven for a disease as complex as shipping fever pneumonia (Hoerlein, 1980; Thomson, 1980; Yates, 1982). Based on Evans' criteria for causation and ideas of Thomson (1980), the etiological significance of different virus infections in shipping fever pneumonia were evaluated (Evans, 1976; Storz et al., 2000a). As a causative factor in the pathogenesis of shipping fever pneumonia, the following criteria have to be met: (1) The viral factor is present in lung tissues and other sites of respiratory tracts of affected cattle. (2) The virus can be isolated in cell cultures at high rates from respiratory samples during the pathogenesis of shipping fever pneumonia. (3) Virus specific immune responses are observed in cattle that recover from shipping fever pneumonia. (4) The virus is not isolated from clinically normal cattle, but it may be detected in cattle with other forms of respiratory tract disease. (5) Cattle with significant levels of antibodies against the virus do not develop shipping fever pneumonia which occurs in cattle without such immune protection in specific epizootics. (6) Elimination of the virus factor prevents or decreases the severity of shipping fever pneumonia.

According to our findings reported in this investigation and other related experiments, we established that all of Evans' criteria of causation were met when

applied to the RBCV infections in the 1997 and 1998 epizootics (Storz et al., 1999; Storz et al., 2000a and b). High rates of RBCV shedding occurred during the early phase of genesis of shipping fever pneumonia among these cattle, and high RBCV titers were detected in their lungs of fatal cases. *Pasteurella haemolytica* was isolated from lung samples. The fatal outcomes of the combined infections of lungs with RBCV and *P. haemolytica* were influenced by the bacterial components which induced terminal necrotizing lesions. The RBCV shedding subsided during the convalescent phase, and treatment with antibiotics accelerated recovery of some of the cattle. Our findings furnish strong support for the conclusion that RBCV played an etiological role in the pathogenesis of shipping fever pneumonia which was previously not recognized, and represents a virtual missing link in the pathogenesis of this disease.

Future investigation should be aimed: (1) to further compare the structural and functional differences of structural and nonstructural genes and their products between the RBCV and EBCV strains, and relate them to the tissue specificity of RBCV; (2) to study the local, systemic, humoral as well as cell-mediated immune responses of cattle to RBCV infection; and (3) to develop effective vaccines based on the immunogenic properties of specific RBCV structural proteins.

REFERENCES

Abraham, S., T.E. Kienzle, W.E. Lapps, and D.A. Brian. 1990a. Sequence and expression analysis of potential nonstructural proteins of 4.9, 4.8, 12.7, and 9.5 kDa encoded between the spike and membrane protein genes of the bovine coronavirus. Virology **17:**488-495.

Abraham, S., T.E. Kienzle, W.E. Lapps, and D.A. Brian. 1990b. Deduced sequence of the bovine coronavirus spike protein and identification of the internal proteolytic cleavage site. Virology **176:**296-301.

Appel, G., H.-P. Heckert, and W. Hofmann. 1992. Über die Beteiligung von bovinem Coronavirus (BCV) am Rindergrippekomplex in Betrieben Schleswig-Holsteins. Tierärztl Umschau **47:**296-304.

Baker, S.C., C.K. Shieh, L.H. Soe, M.F. Chang, D.M. Vannier, and M.M. Lai. 1989. Identification of a domain required for autoproteolyitc cleavage of murine coronavirus gene A polyprotein. J. Virol. **63:**3693-3699.

Baker, S.C., K. Yokomori, S. Dong, R. Carlisle, A.E. Gorbalenya, E.V. Koonin, and M.M. Lai. 1993. Identification of the catalytic sites of a papain-like cysteine proteinase of murine coronavirus. J. Virol. **67:**6056-6063.

Baldwin, D.A., R.G. Marshall, and G.E. Wessman. 1967. Experimental infection of calves with myxovirus parainfluenza-3 and *Pasteurella haemolytica*. Am. J. Vet. Res. **28:**1773-1782.

Ballesteros, M.L., C.M. Sanchez, and L. Enjuanes. 1997. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology **227:**378-388.

Brian, D.A., B.G. Hogue, and T.E. Kienzle. 1995. The coronavirus hemagglutinin esterase glycoprotein, p. 165-179. *In* S.G. Siddell (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.

Briggs, R.E., G.H. Frank, C.W. Purdy, E.S. Zehr, and R.W. Loan. 1998. Rapid spread of a unique strain of *Pasteurella haemolytica* serotype 1 among transported calves. Am. J. Vet. Res. **59:**401-405.

Brown, T.D.K, and I. Brierly. 1995. The Coronavirus non-structural proteins, p. 191-217. *In* S.G. Siddell (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.

Butler, J.E. 1969. Bovine immunoglobulins: a review. J. Dairy Sci. **52:**1895-1909.

Carman, P.S., and M.J. Hazlett. 1992. Bovine coronavirus infection in Ontario, 1990-1991. Can. Vet. J. **33:**812-814.

Cavanagh, D., D.A. Brian, L. Enjuanes, K.V. Holmes, M.M.C. Lai, H. Laude, S.G. Siddell, W. Spaan, F. Taguchi, and P.J. Talbot. 1990. Recommendations of the coronavirus study group for the nomenclature of the structural proteins, mRNAs, and genes of coronaviruses. Virology **176:**306-307.

Cavanagh, D., and M.C. Horzinek. 1993. Genus Torovirus assigned to the Coronaviridae [news]. Arch. Virol. **128:**395-396.

Cereijido, M., E.S. Robbins, W.J. Donlan, C.A. Rotunno, and D.D. Sabatini. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. J. Cell Biol. **77:**853-880.

Chouljenko, V.N., K.G. Kousoulas, X.Q. Lin, and J. Storz. 1998a. Genetic analysis of respiratory bovine coronavirus strains from fatal pneumonia of cattle, p. 149 *In* Proceedings of the Seventy-ninth Conference of Research Workers on Animal Diseases, Chicago, Il.

Chouljenko, V.N., K.G. Kousoulas, X.Q. Lin, and J. Storz. 1998b. Nucleotide and predicted amino acid sequences of all genes encoded by the 3' genomic portion (9.5kb) of respiratory bovine coronaviruses and comparisons among respiratory and enteric coronaviruses. Virus Genes **17:**33-42.

Ciszewski, D.K., J.C. Barker, R.F. Slocombe, J.F. Reindel, D.M. Haines, and E.G. Clark. 1991. Experimental reproduction of respiratory tract disease with bovine respiratory syncytial virus. Vet. Microbiol. **28:**39-60.

Clark, M.A. 1993. Bovine coronavirus. Br. Vet. J. **149:**51-70.

Collier, J.R. 1968. Significance of bacteria in bovine respiratory disease. J. Am. Vet. Med. Assoc. **153:**1645-1651.

Cox, G.J., M.D. Parker, and L.A. Babiuk. 1989. The sequence of cDNA of bovine coronavirus 32K nonstructural gene. Nucleic Acids Research. **17:**5847.

Cox, G.J., M.D. Parker, and L.A. Babiuk. 1991. Bovine coronavirus nonstructural protein ns32 is a phosphoprotein. Virology **185:**509-512.

Craig, R.A., and S. Kapil. 1994. Detection of novel enteric viruses in Wisconsin livestock. *In*: the American Association for Veterinary Laboratory Diagnosticians, Grand Rapids, Mich.

Cunningham, J.G. 1997. Nonrespiratory functions of the lung, p. 603-607. *In* J.G. Cunningham (ed.), Textbook of Veterinary Physiology (2nd edition). W.B. Saunders Company, Philadelphia, Pa.

Delmas, B., and H. Laude. 1990. Assembly of coronavirus spike protein into trimers and its role in epitope expression. J. Virol. **64:**5367-5375.

den Boon, J.A., E.J. Snijder, E.D. Chirnside, A.A. de Vries, M.C. Horzinek, and W.J. Spaan. 1991. Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. J. Virol. **65:**2910-2920.

Denison, M.R., and S. Perlman. 1986. Translation and processing of mouse hepatitis virus virion RNA in a cell-free system. J. Virol. **60:**12-18.

Denison, M.R., and S. Perlman. 1987. Identification of putative polymerase gene product in cells infected with murine coronavirus A59. Virology **157:**565-568.

Denison, M.R., P.W. Zoltick, S.A. Hughes, B. Giangreco, A.L. Olson, S. Perlman, J.L. Leibowitz, and S.R. Weiss. 1992. Intracellular processing of the N-terminal ORF 1a proteins of the coronavirus MHV-A59 requires multiple proteolytic events. Virology **189:**274-284.

Denison, M.R., P.W. Zoltick, J.L. Leibowitz, C.J. Pachuk, and S.R. Weiss. 1991. Identification of polypeptides encoded in open reading frame 1b of the putative polymerase gene of the murine coronavirus mouse hepatitis virus A59. J. Virol. **65:**3076-3082.

Deregt, D., G.A. Gifford, M.K. Ijaz, T.C. Watts, J.E. Gilchrist, D.M. Haines, and L.A. Babiuk. 1989. Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstration of in vivo virus-neutralizing activity. J. Gen. Virol. **70:**993-998.

Deregt, D., M. Sabara, and L.A. Babiuk. 1987. Structural proteins of bovine coronavirus and their intracellular processing. J. Gen. Virol. **68:**2863-2877.

Doughri, A.M., and J. Storz. 1977. Light and ultrastructural pathological changes in intestinal coronavirus infection of newborn calves. *Zentralbl Veterinaermed* [B] **24:**367-385.

Doughri, A.M., J. Storz, I. Hajer, and H.S. Fernando. 1976. Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Exp. Mol. Pathol. **25:**355-370.

Dubois-Dalcq, M.E., E.W. Doller, M.V. Haspel, and K.V. Holmes. 1982. Cell tropism and expression of mouse hepatitis virus (MHV) in mouse spinal cord cultures. Virology **119:**317-331.

Duncan, J.R., B.N. Wilkie, F. Hiestand, and A.J. Winter. 1972. The serum and secretory immunoglobulins of cattle: characterization and quantitation. J. Immunol. **108:**965-976.

Estes, D.M., N.M. Closser, and G.K. Allen. 1994. IFN-γ stimulates IgG2 production from bovine B cells costimulated with anti-μ and mitogen. Cell Immunol. **154:**287-295.

Estes, D.M., A. Hirano, V.K. Heussler, D.A.E. Dobbelaere, and W.C. Brown. 1995. Expression and biological activities of bovine interleukin 4: effects of recombinant bovine interleukin 4 on T cell proliferation and B cell differentiation and proliferation in vitro. Cell Immunol. **163:**268-279.

Evans, A.S. 1976. Causation and disease: The Henle-Koch postulates revisited. Yale J. Biol. Med. **49:**175-195.

Fosmire, J.A., K. Hwang, and S. Makino. 1992. Identification and characterization of a coronavirus packaging signal. J. Virol. **66:**3522-3530.

Frank, G.H., and R.E. Briggs. 1992. Colonization of the tonsils of calves with *Pasteurella haemolytica.* Am. J. Vet. Res. **53:**481-484.

Frank, G.H., R.E. Briggs, and K.G. Gillette. 1986. Colonization of the nasal passages of calves with *Pasteurella haemolytica* serotype 1 and regeneration of colonization after experimentally induced viral infection of the respiratory tract. Am. J. Vet. Res. **47:**1704-1707.

Frank, G.H., and P.C. Smith. 1983. Prevalence of *Pasteurella haemolytica* in transported calves. Am. J. Vet. Res. **44:**981-985.

Frank, G.H., and G.E. Wessman. 1978. Rapid plate agglutination procedure for serotyping. J. Clin. Microb. **7:**142-145.

Friend, S.C.E., R.G. Thomson, and B.N. Wilkie. 1977. Pulmonary lesions induced by *Pasteurella haemolytica* in cattle. Can. J. Comp. Med. **41:**212-223.

Fuller, S., C.-H. von Bonsdorff, and K. Simons. 1984. Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line MDCK. Cell (Cambridge, Mass.) **38:**65-77.

Gottschalk, A. 1957. The specific enzyme of influenza virus and *Vibrio cholerae.* Biochem. Biophys. Acta. **23:**645-646.

Griffiths, G., and P. Rottier. 1992. Cell biology of viruses that assemble along the biosynthetic pathway. Semin. Cell Biol. **3:**367-381.

Guidry, A.J., L.M. Berning, and C.N. Hambleton. 1993. Opsonization of *Staphylococcus aureus* by bovine immunoglobulin isotypes. J. Dairy Sci. **76:**1285-1289.

Hausman, J., E. Kretzschmar, M. Ohuchi, W. Garten, and H.D. Klenk. 1993.. N1 neuraminidase of influenza virus A/FPV/Rostock/34 has hemagglutinin activity. p. 34 *In* Proceedings of the twelfth Annual Meeting American Society for Virology, Davis, Calif.

Heckert, R.A., L.J. Saif, K.H. Hoblet, and A.G. Agnes. 1990. A longitudinal study of bovine coronavirus enteric and respiratory infections in dairy calves in two herds in Ohio. Vet. Microb. **22:**187-201.

Heckert, R.A., L.J. Saif, J.P. Mengel, and G.W. Myers. 1991. Isotype-specific antibody responses to bovine coronavirus structural proteins in serum, feces, and mucosal secretions from experimentally challenge-deprived calves. Am. J. Vet. Res. **52:**692-699.

Herbst, V.W., E. Klatt, and T. Schliesser. 1989. Serologisch-diagnostische Untersuchungen zum Vorkommen von Coronavirusinfektionen bei Atemwegserkrankungen des Rindes. Berl Munch Tieraztl Wochenschr **102:**129-131.

Herrler, G., I. Durkop, H. Becht, and H.-D. Klenk. 1988. The glycoprotein of influenza C virus is the haemagglutinin, esterase and fusion factor. J. Gen. Virol. **69:**839-846.

Herrler, G., and H.-D. Klenk. 1987. The surface receptor is a major determinant of the cell tropism of influenza C virus. Virology. **159:**102-108.

Herrler, G., R. Rott, H.-D. Klenk, H.D. Muller, A.K. Shukda, and R. Schauer. 1985. The receptor-destroying enzyme of influenza C virus: neuraminidate-O-acetyl-esterase. EMBO J. **4:**1503-1506.

Herrler, G, S. Szepanski, and B. Schultze. 1991. 9-O-acetylated sialic acid, a receptor determinant for influenza C virus and coronaviruses. [Review]. Behring Institute Mitteilungen. **1:**177-184.

Hirst, G.K. 1950. The relationship of a new strain of virus to those of the mumps-NDV-influenza group. J. Exp. Med. **91:**177-185.

Hoerlein, A.B. 1980. Shipping fever, p. 99–160. *In* H.E. Amstutz (ed.), Bovine Medicine and Surgery. American Veterinary Publications Inc. Santa Barbara, Calif.

Hoerlein, A.B., S.P. Saxena, and M.E. Mansfield. 1961. Studies on shipping fever of cattle. II. Prevalence of *Pasteurella species* in nasal secretions from normal calves and calves with shipping fever. Am. J. Vet. Res. **122:**470-472.

Hofmann, M.A., R.Y. Chang, S. Ku, and D.A. Brian. 1993. Leader-mRNA junction sequences are unique for each subgenomic mRNA species in the bovine coronavirus and remain so throughout persistent infection. Virology. **196:**163-171.

Hogue, B.G., T.E. Kienzle, and D.A. Brian. 1989. Synthesis and processing of the bovine enteric coronavirus hemagglutinin protein. J. Gen. Virol. **70:**345-352.

Hogue, B.G., B. King, and D.A. Brian. 1984. Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. J. Virol. **51:**384-388.

Hsu, M.-C., A. Scheid, and P.W. Choppin. 1982. Enhancement of membrane fusion activity of Sendai virus by exposure of the virus to basic pH is correlated with a conformational change in the fusion protein. Proc. Nat. Acad. Sci. USA **79:**5862-5866.

Hussain, K.A., J. Storz, and K.G. Kousoulas. 1991. Comparison of bovine coronavirus antigens: monoclonal antibodies to the spike glycoprotein distinguish between vaccine and wild-type stains. Virology **183:**442-445.

Jensen, R., R.E. Pierson, P.M. Braddy, and D.A. Sarri. 1976. Shipping fever pneumonia in yearling feedlot cattle. J. Am. Vet. Med. Assoc.**169:**500-506.

Jimenez, C., W. Herbst, U. Biermannn, M. Muller, and T. Schliesser. 1989. Isolierung von Coronaviren in der Zellkultur aus Nasentupferproben atemwegskranker Kalber in der Bundesrepublik Deutschland. Zentralbl Veterinarmed [B] **36:**635-638.

Jones, L.V., R.W. Compans, A.R. Davis, T. Bos, and D.P. Nayak. 1985. Surface expression of influenza virus neuraminidase, an amino-terminal anchored viral membrane glycoprotein, in polarized epithelial cells. Mol. Cell Biol. **5:**2182-2189.

Kienzle, T.E., S. Abraham, B.G. Hogue, and D.A. Brian. 1990. Structure and orientation of expressed bovine coronavirus hemagglutinin-esterase protein. J. Virol. **64:**1834-1838.

Kim, Y.N., M.M.C. Lai, and S. Makino. 1993. Generation and selection of coronavirus defective interfering RNA with large open reading frame by RNA recombination and possible editing. Virology **194:**244-253.

Kimman, T.G., M.R. Daha, J.M.A. Brinkhof, and F. Westenbrink. 1989a. Activation of complement by bovine respiratory syncytial virus-infected cells. Vet. Immunol. Immunopathol. **21:**311-325.

Kimman, T.G., G.K. Terpstra, M.R. Daha, and F. Westenbrink. 1989b. Pathogenesis of naturally acquired bovine respiratory syncytial virus infection in calves: evidence for the involvement of complement and mast cell mediators. Am. J. Vet. Res. **5**:694-700.

King, B., B.J. Potts, and D.A. Brian. 1985. Bovine coronavirus hemagglutinin protein. Virus Res. **2**:53-59.

Klenk, E., H. Faillard, and H. Lempfrid. 1955. Über die enzymatische Wirkung von Influenza-Virus. Z. Physiol. Chem. **301**:235-246.

Krempl, C., B. Schultze, H. Laude, and G. Herrler. 1997. Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. J. Virol. **71**:3285-3287.

Lai, M.M.C. 1990. Coronavirus: organization, replication and expression of genome. Annu. Rev. Microb. **44**:303-333.

Laporte, J., R. L'Haridon, and P. Bobulesco. 1979. In vitro culture of bovine enteric coronavirus (BEC). Inst. Nat. Sante. Tech. Med. Colloq. **90**:99-102.

Laude, H., and P.S. Masters. 1995. The coronavirus nucleocapsid protein, p. 141-163. *In* S.G. Siddell (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.

Laver, W.G., P.M. Colman, R.G. Webster, V.S. Hinshaw, and G.M. Air. 1984. Influenza virus neuraminidase with hemagglutinin activity. Virology **137**:314-323.

Leibowitz, J.L., S.R. Weiss, E. Paavola, and C.W. Bond. 1982. Cell-free translation of murine coronavirus RNA. J. Virol. **43**:905-913.

LeJan, C., and J. Asso. 1980. The local and systemic immune response of calves following experimental infection with IBR virus. p. 677-692. *In* J.E. Butler (ed.), The Ruminant Immune System. Plenum Press, New York, N.Y.

Lin, S.H., and M.M.C. Lai. 1993. Deletion mapping of a mouse hepatitis virus defective interfering RNA reveals the requirement of an internal and discontiguous sequence for replication. J. Virol. **67**:6110-6118.

Lin, X.Q., M. Burrell, and J. Storz. 1995. Hemagglutinin and receptor destroying functions of newly recognized bovine respiratory coronaviruses, abstr. 4/27. *In* Annual Meeting of American Society for Microbiology-South Central Branch, Little Rock, Ak.

Lin, X.Q., V.N. Chouljenko, K.G. Kousoulas, and J. Storz. 1997a. Functional hemagglutinin-esterase differences in enteric and respiratory bovine coronaviruses, p. 151. *In* Proceedings of the Seventy-eighth Conference of Research Workers on Animal Diseases, Chicago, Il.

Lin, X.Q., K.L. O'Reilly, and J. Storz. 1997b. Infection of polarized epithelial cells with enteric and respiratory tract bovine coronaviruses and release of virus progeny. Am. J. Vet. Res. **58:**1120-1124.

Loan, R.W., C.W. Purdy, and M.G. Tigges. 1988. A tissue culture-derived *Pasteurella haemolytica* vaccine, p. 165-170. *In* Proceedings of the fifteenth World Congress on the Diseases of Cattle, Palma de Malorca, Spain.

Locker, J.K., J.K. Rose, M.C. Horzinek, and P.J. Rottier. 1992. O-glycosylation of the coronavirus M protein. Differential localization of sialyltransferases in N- and O-linked glycosylation. J. Biol. Chem. **267:**14094-1401.

Luytjes W, P.J., Bredenbeek, A.F. Noten, M.C. Horzinek, and W.J. Spaan. 1988. Sequence of mouse hepatitis virus A59 mRNA 2: Indications for RNA recombination between coronaviruses and influenza C virus. Virology **166:**415-422.

Machamer, C.E., S.A. Mentone, J.K. Rose, and M.G. Farquhar. 1990. The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex. Proc. Natl. Acad. Sci. USA **87:**6944-6948.

Machamer, C.E., and J.K. Rose. 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. J. Cell Biol. **105:**1205-1214.

Madin, S.H., C.F. York, and D.G. McKercher. 1956. Isolation of the infectious bovine rhinotracheitis virus. Science **124:**721-722.

Magwood, S.E., D.A. Barnum, and R.G. Thomson. 1969. Nasal bacterial flora of calves in healthy and in pneumonia-prone herds. Can. J. Comp. Med. **33:**237-243.

Makino, S., and M.M.C. Lai. 1989. High-frequency leader sequence switching during coronavirus defective interfering RNA replication. J. Virol. **63:**5285-5292.

Martin, S.W. 1985. Analysis and causal interpretation of biologic data. A seroepidemiologic study of respiratory disease. Fourth International Symposium on Veterinary Epidemiology and Economics. Vet. Med. **57:**46-54.

Masters, P.S., C.A. Koetzner, C.A. Kerr, and Y. Heo. 1994. Optimization of targeted RNA recombination and mapping of a novel nucleocapsid gene mutation in the coronavirus mouse hepatitis virus. J. Virol. **68:**328-337.

McGuire, T.C, and A.J. Musoke. 1980. Biologic activities of bovine IgG subclasses. p. 359-366. *In* J.E. Butler (ed.), The Ruminant Immune System. Plenum Press, New York, N.Y.

McGuire, T.C, A.J. Musoke, and T. Kurtti. 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology **38:**249-256.

McGuire, T.C., N.D. Pfeiffer, J.M. Weikel, and R.C. Bartsch. 1976. Failure of colostral immunoglobulin transfer in calves dying from infectious disease. J. Am. Vet. Med. Assoc. **169:**713-718.

McKercher, D.G., J.E. Moulton, S.H. Madin, and J.W. Kendrick. 1957. Infectious bovine rhinotracheitis - A newly recognized virus disease of cattle. Am. J. Vet. Res. **18:**246-256.

McNulty, M.S., D.G. Bryson, G.M. Allan and F.F. Logan. 1984. Coronavirus infection of the bovine respiratory tract. Vet. Microb. **9:**425-434.

Mebus, C.A., E.L. Stair, M.B. Rhodes, and M.J. Twiehaus. 1973. Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a corona-like agent. Am. J. Vet. Res. **34:**145-150.

Misfeldt, D.S., S.T. Hamamoto, and D.R. Pitelka. 1976. Transepithelial transport in cell culture. Proc. Natl. Acad. Sci. USA **73:**1212-1216.

Mollenhauer, H.H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. **39:**111-114.

Möstl, V.K., and F. Bürki. 1988. Ursächliche Beteiligung boviner Coronaviren an respiratorischen Krankheitsausbrüchen bei Kälbern und pathogenetisch-immunologische Überlegungen hierzu. Dtsch. Tierärztl. Wochenschr. **95:**19-22.

Murphy, J.S., and F.B. Bang. 1952. Observations with electron microscope on cells of chick chorioallantoic membrane infected with influenza virus. J. Exp. Med. **95:**259-268.

Murphy, B.R., and R.G. Webster. 1996. Orthomyxoviruses,, p. 1397-1445. *In* B.N. Fields et al. (ed.), Fields Virology (3rd edition). Lippincott-Raven Publishers, Philadelphia, Pa.

Nansen, P. 1970. Metabolism of bovine immunoglobulin-G. Thesis Royal Veterinary and Agricultural University, Munksgaard, Copenhagen.

Opstelten, D.J., P. deGroote, M.C. Horzinek, H. Vennema, and P.J. Rottier. 1993. Disulfide bonds in folding and transport of mouse hepatitis coronavirus glycoproteins. J. Virol. **67:**7394-7401.

Palese, P., K. Tobita, M. Ueda, and R.W. Compans. 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology **61:**397-410.

Parker, M.D., G.J. Cox, D. Deregt, D.R. Fitzpatrick, and L.A. Babuik. 1989. Cloning and in vitro expression of the gene for the E3 haemagglutinin glycoprotein of bovine coronavirus. J. Gen. Virol. **70:**155-164.

Parker, M.D., D. Yoo, and L.A. Babiuk. 1990. Expression and secretion of bovine coronavirus hemagglutinin-esterase glycoprotein by insect cells infected with recombinant baculoviruses. J. Virol. **64:**1625-1629.

Payne, H.R., and J. Storz. 1990. Scanning electron microscopic characterization of bovine coronavirus plaques. J. Vet. Med. [B] **37:**501-508.

Payne, H.R., J. Storz, and W.G. Henk. 1990a. Bovine coronavirus antigen in the host cell plasmalemma. Exp. Mol. Path. **53:**152-159.

Payne, H.R., J. Storz, and W.G. Henk. 1990b. Initial events in bovine coronavirus infection: analysis through immunogold probes and lysosomotropic inhibitors. Arch. Virol. **114:**175-189.

Potgieter, L.N.D. 1975. The influence of complement on the neutralization of IBR by globulins derived from early and late bovine antisera. Can. J. Comp. Med. **39:**427-433.

Potgieter, L.N.D., M.D. McCracken, F.M. Hopkins, R.D. Walker, and J.S. Guy. 1984. Experimental production of bovine respiratory tract disease with bovine viral diarrhea virus. Am. J. Vet. Res. **45:**1582-1585.

Rasschaert, D., M. Duarte, and H. Laude. 1990. Porcine respiratory coronavirus differs from transmissible gastroenteritis virus by a few genomic deletions. J. Gen. Virol. **71:**2599-2607.

Ratafia, M. 1988. Genetically engineered vaccines: World business opportunities. Am. Clin. Prod. Rev. **7:**18-21.

Reisinger, R.C., K.L. Heddleston, and C.A. Manthei. 1959. A myxovirus (SF-4) associated with shipping fever of cattle. J. Am. Vet. Med. Assoc. **135:**147-154.

Robbins, S.G., M.F. Frana, J.J. McGowan, J.F. Boyle, and K.V. Holmes. 1986. RNA-binding proteins of coronavirus MHV: detection of monomeric and multimeric N protein with an RNA overlay-protein blot assay. Virology **150:**402-410.

Rodriguez-Boulan, E., and D.D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc. Natl. Acad. Sci. USA **75:**5071-5075.

Rosenquist, B.D. 1974. Isolation of respiratory syncytial virus from calves with acute respiratory disease. J. Inf. Dis. **130:**177-182.

Rossen, J.W.A., C.P.J. Bekker, W.F. Voorhout, M.C. Horzinek, A. Van Der Ednde, G.J.A.M. Strous, and P.J.M. Rottier. 1995. Coronaviruses in polarized epithelial cells, p. 135-138. *In* P.J. Talbot, and G.A. Levy (ed.), Corona- and Related Viruses. Plenum Press, New York, N.Y.

Rossi, C.R., and G.K. Kiesel. 1976. Antibody class and complement requirement of neutralizing antibodies in the primary and secondary response of cattle to IBR vaccines. Arch. Virol. **51:**191-196.

Roth, M.G., R.W. Compans, L. Giusti, A.R. Davis, D.D. Nayak, M.J. Gething, and J.S. Sambrook. 1983. Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV_{40} viruses carrying cloned hemagglutinin DNA. Cell (Cambridge, Mass.) **33:**435-443.

Rott, R. 1979. Molecular basis of infectivity and pathogenicity of myxovirus. Arch. Virol. **59:**285-298.

Rottier, P.J.M. 1995. The coronavirus membrane glycoprotein, p. 115-139. *In* S.G. Siddell (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.

Saif, L.J., D.R. Redman, K.V. Brock, E.M. Kohler, and R.A. Heckert. 1988. Winter dysentery in adult dairy cattle: detection of coronavirus in the feces. Vet. Rec. **123:**300-301.

Saif, L.J., D.R. Redman, P.D. Moorhead, and K.W. Theil. 1986. Experimentally induced coronavirus infections in calves: viral replication in the respiratory and intestinal tracts. Am. J. Vet. Res. **7:**1426-1432.

Sawicki, S.G., and D.L. Sawicki. 1990. Coronavirus transcription: subgenomic mouse hepatitis virus replicative intermediates function in RNA synthesis. J. Virol. **64:**1050-1056.

Schmidt, M.F.G. 1982. Acylation of viral spike glycoproteins, a feature of enveloped RNA viruses. Virology **116:**327-338.

Schmidt, O.W., and G.E. Kenny. 1982. Polypeptides and functions of antigens from human coronaviruses 229E and OC43. Infect. Immun. **32:**1000-1006.

Schultze, B., H.J. Gross, R. Brossmer, and G. Herrler. 1991a. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-O-acetylated sialic acid as a receptor determinant . J. Virol. **65:**6232-6237.

Schultze, B., and G. Herrler. 1992. Bovine coronavirus uses N-acetyl-9-O-acetyl-neuraminic acid as a receptor determinant to initiate the infection of cultured cells. J. Gen. Virol. **74:**901-906

Schultze, B., and G. Herrler. 1995. Polarized entry of bovine coronavirus in epithelial cells, p. 375-378. *In* P.J. Talbot, and G.A. Levy (ed.), Corona- and Related Viruses. Plenum Press, New York, N.Y.

Schultze, B., K. Wahn, H.D. Klenk,, and G. Herrler. 1991b. Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity. Virology **180:**221-228.

Sethna, P.B., M.A. Hofmann, and D.A. Brian. 1991. Minus-strand copies of replicating coronavirus mRNAs contain antileaders. J. Virol. **65:**320-325.

Sethna, P.S., S.L. Hung, and D.A. Brian. 1989. Coronavirus subgenomic minus-strand RNAs and the potential for mRNA replicons. Proc. Natl. Aca. Sci. USA **86:**5626-5630.

Shieh, C.K., H.J., Lee, K. Yokomori, N. La Monica, S. Makino, and M.M.C. Lai. 1989. Identification of a new transcriptional initiation site and the corresponding functional gene 2b in the murine coronavirus RNA genome. J. Virol. **63:**3729-3736.

Shieh, C.K., L.H. Soe, S. Makino, M.F. Chang, S.A. Stohlman, and M.M.C. Lai. 1987. The 5'-end sequence of the murine coronavirus genome: implications for multiple fusion sites in leader-primed transcription. Virology **156:**321-330.

Siddell, S. 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. J. Gen. Virol. **64:**113-125.

Snijder, E.J., J.A. den Boon, M.C. Horzinek, and W.J. Spaan. 1991. Comparison of the genome organization of toro- and coronaviruses: evidence for two nonhomologous RNA recombination events during Berne virus evolution. Virology **180:**448-452.

Spaan, W., D. Cavanagh, and M.C. Horzinek. 1988. Coronavirus: structure and genome expression. J. Gen. Virol. **69:**2939-2952.

St. Cyr-Coats, K., H.R. Payne, and J. Storz. 1988a. The influence of the host cell and trypsin treatment on bovine coronavirus infectivity. J. Vet. Med. **35:**752-759.

St. Cyr-Coats, K., and J. Storz. 1988. Bovine coronavirus-induced cytopathic expression and plaque formation: host cell and virus strain determine trypsin dependence. J. Vet. Med. [B] **35:**48-56.

St. Cyr-Coats, K., J. Storz, K.A. Hussain, and K.L. Schnorr. 1988b. Structural proteins of bovine coronavirus strain L9: effects of the host cell and trypsin treatment. Arch. Virol. **103:** 35-45.

Stephens, E.B., and R.W. Compans. 1986. Nonpolarized expression of a secreted murine leukemia virus glycoprotein in polarized epithelial cells. Cell (Cambridge, Mass.) **47:**1053-1059.

Stohlman, S.A., R.S. Baric, G.N. Nelson, L.H. Soe, L.M. Welter, and R.J. Deans. 1988. Specific interaction between coronavirus leader RNA and nucleocapsid protein. J. Virol. **62:**4288-4295.

Stohlman, S.A., J.O. Fleming, C.D. Patton, and M.M.C. Lai. 1983. Synthesis and subcellular localization of the murine coronavirus nucleocapsid protein. Virology **130:**527-532.

Stohlman, S.A., S. Kyuwa, J.M. Polo, D. Brady, M.M.C. Lai, and C.C. Bergmann. 1993. Characterization of mouse hepatitis virus-specific cytotoxic T cells derived from the central nervous system of mice infected with the JHM strain. J. Virol. **67:**7050-7059.

Storz, J. 1998. Respiratory disease of cattle associated with coronavirus infections. *In* J.L. Howard, and R.A. Smith (ed.), p. 291-293. Current Veterinary Therapy: Food Animal Practice 4. WB Saunders Co, Philadelphia, Pa.

Storz, J., G. Herrler, D.R. Snodgrass, K.A. Hussain, X.M. Zhang, M.A. Clark, and R. Rott. 1991. Monoclonal antibodies differentiate between the haemagglutinating and the receptor–destroying activities of bovine coronavirus. J. Gen. Virol. **72:**2817-2820.

Storz, J., X.Q. Lin, C.W. Purdy, V.N. Chouljenko, K.G. Kousoulas, F.M. Enright, W.C. Gilmore, R.E. Corstvet, and R.W. Loan. 2000a. Combined infections with coronaviruses and *Pasteurella spp.* in bovine shipping fever pneumonia and Evans' criteria of causation. Infect. Immun. Submitted for publication.

Storz, J., X.Q. Lin, C.W. Purdy, and R.W. Loan. 1999. Novel diagnostics for defining virus infections in shipping fever pneumonia: emergence of respiratory bovine coronaviruses, p 54-60. *In* Proceedings of the fourth international symposium on world association of veterinary laboratory diagnosticians, College Station, Tx.

Storz, J., C.W. Purdy, X.Q. Lin, M. Burrell, R.E. Truax, R.E. Briggs, G.H. Frank, and R.W. Loan. 2000b. Isolation of coronaviruses and *Pasteurella spp.* from cattle involved in 2 outbreaks of shipping fever. J. Am. Vet. Med. Assoc. In press.

Storz, J., und R. Rott. 1980. Über die Verbreitung der Coronavirusinfektion bei Rindern in ausgewählten Gebieten Deutschlands: Antikörpernachweis durch Mikroimmundiffusion und Neutralisation. Dtsch. Tierärztl. Wschr. **87:**252-254.

Storz, J., and R. Rott. 1981. Reactivity of antibodies in human serum with antigens of an enteropathogenic bovine coronavirus. Med. Microb. Immun. **169:**169-178.

Storz, J., R. Rott, and G. Kaluza. 1981. Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infect. Immun. **31:**1214-1222.

Storz, J., L. Stine, A. Liem, and G.A. Anderson. 1996. Coronavirus isolation from nasal swab samples in cattle with signs of respiratory tract disease after shipping. J Am. Vet. Med. Assoc. **208:**1452-1455.

Storz, J., and X.M. Zhang. 1991. Coronaviruses and enteropathogenicity: infectious mechanisms of enteropathogenic bovine coronaviruses. Behring Inst. Mitt. **89:**130-137.

Storz, J., X.M. Zhang, and R. Rott. 1992. Comparison of hemagglutinating, receptor-destroying, and acetylesterase activities of avirulent and virulent bovine coronavirus strains. Arch. Virol. **125:**193-204.

Strobl, B., and R. Vlasak. 1993. The receptor-destroying enzyme of influenza C virus is required for entry into target cells. Virology **192:**679-682.

Sturman, L.S., and K.V. Holmes. 1983. The molecular biology of coronaviruses. Adv. Virus Res. **28:**35-112.

Sturman, L.S., C.S. Ricard, and K.V. Holmes. 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. J. Virol. **56:**904-911.

Swift, A.M., and C.E. Machamer. 1991. A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. J. Cell Biol. **115:**19-30.

Thomson, R.G. 1980. A perspective on respiratory disease of feedlot cattle. Can. Vet. J. **21:**181-185.

Tompkins, W.A.F., A.M. Watrach, J.D. Schmale, R.M. Schultz, and J.A. Harris. 1974. Cultural and antigenic properties of newly established cell strains from adenocarcinomas of human colon and rectum. J. Natl. Cancer Inst. **52:**101-106.

Tooze, J., and S.A Tooze. 1985. Infection of AtT20 murine pituitary tumor cells by mouse hepatitis virus strain A59: virus budding is restricted to the Golgi region. Eur. J. Cell Bio. **37:**203-212.

Tooze, J., S.A. Tooze, and G. Warren. 1984. Replication of coronavirus MHV-A59 in sac- cells: determination of the first site of budding of progeny virions. Eur. J. Cell Bio. **33:**281-293.

Tucker, S.P., and R.W. Compans. 1993. Virus infection of polarized epithelial cells. Adv. Virus Res. **42:**187-247.

van der Most, R.G., P.J. Bredenbeek, and W.J. Spaan. 1991. A domain at the 3' end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. J. Virol. **65:**3219-3226.

van der Most, R.G., and W.J. Spaan. 1995. Coronavirus replication, transcription and RNA recombination, p. 11-31. *In* S.G. Siddell (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.

Vennema, H., L. Heijnen, A. Zijderveld, M.C. Horzinek, and W.J. Spaan. 1990. Intracellular transport of recombinant coronavirus spike proteins: implications for virus assembly. J. Virol. **64:**339-346.

Vlasak, R., W. Luytjes, J. Leider, W. Spaan, and P. Palese. 1988a. The E3 protein of bovine coronavirus is a receptor–destroying enzyme with acetylesterase activity. J. Virol. **62:**4686-4690.

Vlasak, R., W. Luytjes, W. Spaan, and P. Palese. 1988b. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proc. Natl. Acad. Sci. USA **85:**4526-4529.

Vlasak, R., T. Muster, A.M. Lauro, J.C. Powers, and P. Palese. 1989. Influenza C virus esterase: analysis of catalytic site, inhibition, and possible function. J. Virol. **63:**2056-2062.

Weaber, D. 1998. The Statistics. Natl. Cattleman. **13(4):** 73-76.

Weaver, R.E., and D.G. Hollins. 1980. Gram-negative fermentative bacteria and *Franchisella tularensis*, p. 242-262. *In* E.H. Lennette, A. Balows, W.J. Hausler, Jr., and J.P. Traut (ed.), Manual of Clinical Microbiology, 3rd ed. Am. Soc. Microb., Washington, D.C.

Wege, H., S. Siddell, and V. terMeulen. 1982. The biology and pathogenesis of coronaviruses. [Review]. Current Topics in Microbiology & Immunology. **99:**165-200.

Whitely, L.O., S.K. Maheswaran, D.J. Weiss, T.R. Ames, and M.S. Kannan.. 1992. *Pasteurella haemolutica* A1 and bovine respiratory disease:pathogenesis. J. Vet. Intern. Med. **6:**11-22.

Williams, M.R., and R.L. Spooner. 1975. Quantitative studies on bovine immunoglobulins. Vet. Rec. **96:**81-84.

Williams, J.R. 1983. Proteolytic Alteration of Bovine Coronavirus Biological and Physical Properties. Dissertation Department of Microbiology and Environmental Health, Colorado State University, Fort Collins, Co.

Worku, M., M.J. Paape, and W.W. Marquardt. 1994. Modulation of Fc receptors for IgG on bovine polymorphonuclear neutrophils by interferon-γ through de novo RNA transcription and protein synthesis. Am. J. Vet. Res. **55:**234-238.

Yates, W.D.G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. **46:**225-263.

Yokomori, K., N. La Monica, S. Makino, C.K. Shieh, and M.M.C. Lai. 1989. Biosynthesis, structure, and biological activities of envelope protein gp65 of murine coronavirus. Virology **173:**683-691.

Yoo, D.W., F.L. Graham, L. Prevec, M.D. Parker, M. Benko, T. Zamb, and L.A. Babiuk. 1992. Synthesis and processing of the haemagglutinin-esterase glycoprotein of bovine coronavirus encoded in the E3 region of adenovirus. J. Gen. Virol. **73:**2591-2600.

Yoo, D.W., M.D. Parker, and L.A. Babiuk. 1991. The S2 subunit of the spike glycoprotein of bovine coronavirus mediates membrane fusion in insect cells. Virology **180:**395-399.

Zhang, X.M., W. Herbst, K.G. Kousoulas, and J. Storz. 1994. Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrheic child. J. Med. Virol. **44:**152-161.

Zhang, X.M., K.G. Kousoulas, and J. Storz. 1991. The hemagglutinin/esterase glycoprotein of bovine coronaviruses: sequence and function comparison between virulent and avirulent strains. Virology **185:**847-852.

APPENDIX: LETTER OF PERMISSION

AMERICAN VETERINARY MEDICAL ASSOCIATION
1931 N. MEACHAM ROAD, SUITE 100 • SCHAUMBURG, ILLINOIS 60173-4360
PHONE 847-925-8070 FAX 847-925-1329

Tuesday 29 February 2000

Johannes Storz, DVM, PhD
Professor and Department Head
Major Professor for Xiaoqing Lin's Graduate Studies and Research

and

Xiaoqing Lin, MD
Graduate Research Assistant

Dear Drs. Storz and Lin:

We grant permission for Dr. Lin to include the scientific findings of his article (the citation of which appears below) in his dissertation entitled "Isolation and characterization of newly emerging coronaviruses in acute respiratory tract diseases of cattle".

Lin, X; O'Reilly, KL; & Storz, J
Infection of polarized epithelial cells with enteric and respiratory tract bovine coronoviruses and release of virus progeny
American Journal of Veterinary Research, 1997;58:1120-1124

American Veterinary Medical Association
Kurt J Matushek, DVM, MS
Associate Editor

VITA

Xiaoqing Lin was born in Shanghai, People's Republic of China, on February 7, 1966, the daughter of Mr. Songbo Lin and Mrs. Fudi Wang. She received her high school diploma from the High School associated with the East China Normal University, Shanghai, in July, 1984, and earned her medical doctor degree from the Shanghai Medical University, in July, 1990. After graduation, Dr. Lin served as a pathology resident in obstetrics and gynecology from July, 1990, to July, 1993, through the Department of Pathology at the Women and Children Health Hospital for International Peace. Dr. Lin assumed a graduate research assistantship in August, 1993, in the Department of Biochemistry at the Medical Sciences Campus of the University of Puerto Rico, San Juan, Puerto Rico. She investigated amino acids involved in the allosteric regulation in hypoxanthine phosphoribosyltransferase and associated with the formation of functional dimers through studies on enzymatic kinetics and site-directed mutagenesis. She transferred in June, 1995, to the graduate program of study and research in the Department of Veterinary Microbiology and Parasitology, the School of Veterinary Medicine at the Louisiana State University. She investigated newly emerging epizootics of respiratory tract diseases associated with coronavirus infections of cattle. She is scheduled to earn the degree of Doctor of Philosophy in Veterinary Medical Sciences in May, 2000.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Xiaoqing Lin

Major Field: Veterinary Medical Sciences

Title of Dissertation: Isolation and Characterization of Newly Emerging Coronaviruses in Acute Respiratory Tract Diseases of Cattle

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

March 15, 2000